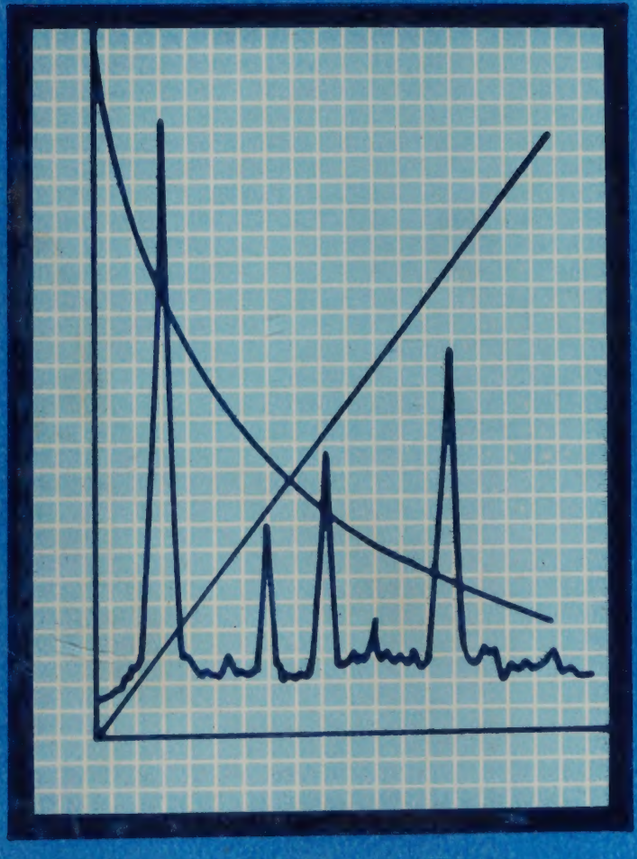
A MANUAL OF LABORATORY TECHNIQUES



NATIONAL INSTITUTE OF NUTRITION
INDIAN COUNCIL OF MEDICAL RESEARCH
HYDERABAD - 500 007 : : INDIA

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A MANUAL OF LABORATORY TECHNIQUES

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PREFACE

A wide range of methods and techniques are used in biomedical research and these methods should be well tested ones with adequate precision and reproducibility. There are also a number of methods for each measurement reported in literature and it poses some difficulty in selecting a suitable method particularly for those who have newly entered the research field. Compilation of appropriate methods for a wide range of measurements employed in biomedical research is most welcome to researchers and technicians and the present manual is an attempt in that direction. The main purpose of this manual is to make available a wide range of analytical and biochemical techniques particularly to those who have a limited access to literature. Several of the methods and techniques included in this manual have evolved out of actual experience of research workers in this Institute with these methods.

As it was considered essential for any one using these methods to be fully conversant with the basic principles of the methods and of the instruments used, the first section (Section A) of the manual has been devoted for describing the broad principles underlying the methods described. The details of techniques and methods have been given in the second section (Section B). Whenever a parameter can be estimated with equal ease and precision by more than one method, alternate techniques have also been given. Use of experimental animals forms an essential part of biomedical research. So, details of handling, feeding and care of animals and specialised techniques useful in animal experimentation are described in some detail in one of the chapters in Section B. Since validity of data obtained in any biological experiment depends to a large extent on the use of proper experimental design and analysis of data, statistical methods appropriate for biological research are exhaustively described in another chapter of Section B. Many of the common laboratory procedures like glassware washing, water purification, and purification of organic solvents etc. are brought together in chapter on 'Miscellaneous methods'. Useful data on preparation of buffers, ion exchange resins, chromatographic media, radioisotopes and liquid scintillation media etc. are given in the Appendix (Section C).

The present attempt to bring out a manual on a wide range of techniques used in biomedical research, nutrition research in particular, would not have been possible without the valuable and expert contributions and active cooperation of several members of staff of the Institute who had had considerable first hand experience with these methods. My thanks are due to these colleagues of mine for their active cooperation in bringing out this manual. I have great pleasure in acknowledging the invaluable help of my young colleagues who have spent much of their valuable time and energy in assembling, editing and bringing out this manual in the present form. I would also like to express my appreciation of the expert help received from the artists and the staff of the printing section of the Institute in the preparation of the cover design and charts for the manual. My grateful thanks are due to Indian Council of Medical Research for financial support in bringing out this manual.

We will consider our effort rewarding, if this manual is widely used by researchers and technicians and find it useful.

B. S. Narasinga Rao
DIRECTOR

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SECTION - A

GENERAL ANALYTICAL TECHNIQUES AND PRINCIPLES

GENERAL ANALYTICAL TECHNIQUES AND PRINCIPLES

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1. COLORIMETRY AND SPECTROPHOTOMETRY

According to the electromagnetic theory of light, light travels in the form of waves described by three attributes – the wavelength, frequency and energy. The visible portion of the electromagnetic spectrum extends from 360-900 nm and the ultraviolet (UV) from 200-380 nm.

Absorption process : Each different atomic and hence molecular structure of a chemical substance has unique resonant frequencies. When the frequency of the incident radiation on a substance coincides with this resonant frequency or energy level, its radiant energy is imparted to the substance. This phenomenon of energy transfer is referred to as absorption. Liquids and other transparent material display colour when they selectively absorb wavelegths of radiant energy in the visible region. Although this effect can be observed by the eye only in the visible region, it exemplifies the absorption process in the neighbouring UV and infra red regions. The amount of radiant energy absorbed by a sample at a certain wavelength depends upon the concentration of the sample.

Beer's law : Beer's law states that the optical density of a solution is directly proportional to the concentration and optical path length through the solution.

If I_o is the intensity of radiation incident on a solution and I is the intensity of the transmitted light, then Transmittance (T) is,

$$T = \frac{I}{I_o}$$

Percentage transmittance = T x 100

The optical density (OD) of a solution is defined as, the logarithm of reciprocal transmittance,

$$\text{ie. } OD = \text{Log} \frac{1}{T} \text{ or } OD = 2 - \log T\ \%$$

According to Beer's law,

OD $\propto$ C x L, where C = concentration of solution, L = path length of light through solution.

$\therefore$ OD = E x C x L, where, E is a proportionality constant called extinction coefficient.

Colorimeter : A colorimeter uses a tungsten lamp as a source of radiation. A filter isolates a rather broad band of wavelengths at which the substances to be estimated absorb maximally. The isolated beam of light then passes through the sample placed in a cuvette of fixed path length where a part of the incident energy is absorbed so that the transmitted beam of light is reduced in intensity in proportion to the concentration of the sample in solution. The transmitted beam of light then falls on a photocell or a phototube which converts the light signal into an electrical signal which is finally read out as transmittance or optical density on a suitable meter.

Spectrophotometer: The spectrophotometer uses a tungsten lamp for measurements in the visible region (360 to 900 nm) and a hydrogen or deuterium lamp for the ultraviolet region (200 to 380 nm) of the spectrum. A monochromator consisting of a grating or a prism or a combination of both is used to obtain a narrow band of wavelengths continuously selectable through the exit slit of the monochromator. The beam of light transmitted by the sample is detected with the help of a suitable detector either a phototube or a photomultiplier tube (for greater sensitivity) and the optical density displayed on a suitable analogue or digital read out.

2. FLUORIMETRY

Fluorescence: It is the immediate emission of light of a longer wavelength from a molecule following the absorption of radiation. When a fluorescent solution is energised by light from a suitable source at relatively shorter wavelengths, this light is absorbed by the sample, which then fluoresces emitting light of higher wavelength.

Fluorimeter: The above principle is used for the quantitation of the amount of fluorescent substance present in solution. A mercury vapour lamp is employed, the wavelength of which is controlled by the choice of suitable filters, known as primary filters. The light emitted from the solution is then passed through a filter, known as the secondary filter and falls on to a suitable detector, the response of which can be read in a meter.

Spectrofluorimeter: This instrument uses a xenon arc lamp which gives a continuous band of light from 200 - 700 nm. Discrete wavelength of 1 nm band width is obtained in primary as well as secondary mohochromater by using diffraction grating. Large number of compounds whose activation and emission spectra are known can be estimated accurately with this instrument.

Measurement: Unlike colorimetry where the scale reads in OD or transmittance, in fluorimeters and spectrofluorimeters the scale is arbitrary and may read continuosly from 1 to 1000 or 1 to 10,000. Quinine sulphate 1 μg/ml in 0.05M H_2SO_4, 1 μg/ml of fluorescin in 0.1 M NaOH, 1 0 μg/ml of anthracen in ethanol (freshly bubbled with oxygen free nitrogen) and 1.0 μg/ml of tyrosine in water can be used as standards and the amount of fluorescence of unknown substances can be compared with them. The results are expressed as relative fluorescence units.

Precautions: Since the arc in the xenon lamp used as the light source may shift, the instrument need to be calibrated each time with the standard solutions suggested above. The fluorescence is profoundly influenced by pH variations.

3. FLAME PHOTOMETRY AND ATOMIC ABSORPTION SPECTROPHOTOMETRY

Flame photometry: Flame photometry is the determination of concentration of an element in solution by measurement of emission of light by atoms of the element in a flame. It is well known that characteristic coloured radiations are produced when salts such as those of sodium, calcium, copper etc., are introduced into a flame. Emission of such characteristic radiation by each element and the

correlation of the emission intensity with the concentration of that element form the basis of flame photometry.

The sample to be analysed is prepared in solution and sprayed under controlled conditions into a flame. The light from the flame enters a monochromator to isolate the desired region of the spectrum. A photocell and an amplifier measure the intensity of the isolated radiation. After proper calibration of the photometer with solutions of known composition and concentration, it is possible to correlate the intensity of a given spectral line with the unknown concentration of an element present.

Whereas flame photometry is best suited for the determination of elements such as sodium, potassium and calcium the sensitivity of this technique for other elements is rather poor. Moreover, reproducibility of readings is dependent on the temperature of the flame There has been a growing need for a sensitive, specific analytical method for trace metal determinations especially in biological systems. Many metals occur together and are often present in concentration of the order of 10^{-6} moles or less. For the determination of a wide number of trace elements today, atomic absorption spectrophotometry is the method of choice.

Atomic absorption spectrophotometry : Atomic absorption spectrophotometry is the determination of concentration of a substance by the measurement of absorption of the characteristic radiation by the atomic vapour of an element. When radiation characteristics of a particular element passes through an atomic vapour of the same element, absorption of radiation occurs in proportion to the concentration of the atoms in the light path. The source of characteristic radiation is a hallow cathode lamp, the cathode being made of the element desired to be estimated. This is an additional element used in atomic absorption spectrophotometer, other components being the same as for a flame photometer.

Where sample size is limited and higher degree of sensitivity is desired flameless atomic absorption spectrophotomety is used. In the flameless method a graphite furnance is used to atomise the sample instead of a flame. The technique is about 20-500 times more sensitive than the flame technique. As little as 5 μl of a sample is enough for most determinations.

4. CHROMATOGRAPHY

Chromatography is by far the most useful general group of techniques available at present for the separation of closely related compounds in a mixture. It is essentially a separation technique. Here the separation is effected by differences in the equilibrium distribution of the components between two immiscible phases, viz., the stationary and the mobile phases. These differences in the equilibrium distribution are a result of the nature and degree of interaction of the components with these two phases. The stationary phase is a porous medium through which the sample mixture percolates under the influence of a moving solvent (the mobile phase). There are a number of interactions between the sample and the stationary phase and these have been well exploited to effect the separation of compounds.

Chromatographic procedures are often named after the main type of interaction between the sample and the stationary phase. Thus we have absorption, partition, ion exchange, molecular exclusion or gelpermeation and affinity chromatography. Based on the mobile phase used, chromatographic systems can be classified as liquid chromatography and gas chromatography. Chromatographic techniques are also frequently classified, based on the stationary phase used, as paper chromatogrhaphy, thin

layer chromatography, column chromatography, gas liquid chromatography and high pressure liquid chromatography.

a. Paper and thin layer chromatography : Paper and thin layer chromatographic (TLC) techniques are the easiest to perform and require simple apparatus. They readily provide qualitative information and with careful attention to details, it is possible to obtain quantitative data. In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is placed near one end of the paper strip or TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is known as development. The strip or plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. The paper or silica gel acts as an inert support, the interstices of which hold the more polar phase (usually aqueous) of the solvent mixture which thus acts as the stationary phase, the less polar phase acting as the mobile phase. Separation results from differences in partition equilibrium of the components in the mixture. However, the paper or silica gel interacts (adsorption and some degree of ion exchange) with the components and this effects the separation.

An important characteristic used in paper and thin layer chromatography for identification of compounds is the 'Rf value'.

$$Rf = \frac{\text{distance moved by the substance from origin}}{\text{distance moved by the solvent front from the origin}}$$

Paper and thin layer chromatographic techniques are immensely versatile and one can readily alter the various parameters to achieve a particular separation. These techniques have therefore become indispensable in biomedical research. There are different ways in which chromatograms are run. They are unidimensional ascending and descending, circular or horizontal and two dimensional.

Ascending chromatography : After the material has been spotted on a paper (15" x 15"), the paper is folded into a cylinderical form and stitched at the joint, at three places. The paper cylinder is saturated with the solvent vapour for 1 to 2 hr before dipping into the solvent system which is kept in a petridish and enclosed by a bell jar. The unit is kept on a clean glass plate and sealed with modelling clay all around to make it air tight. Usually, the solvent is allowed to run overnight. The next day the paper is taken out, the solvent front marked with a lead pencil, air dried to remove last traces of the solvent.

Descending chromatography : This requires the solvent to be kept at the top in a trough and the end of the paper close to the spotting line is dipped into the solvent. The solvent flows downward.

Circular or horizontal chromatography : A circle of 12 inches diameter is cut out from a paper. An inner circle of 1.5–2.0 inches is drawn out by lead pencil and the spots are applied on this circle. A small hole is made at the centre of the paper and a paper wick is inserted into it. The paper after spotting is fitted in such a way that the wick dips into the solvent kept in a small petridish and the edges are supported on the corks. This technique has the advantage that the run is short (about 4 to 6 hr) and the disadvantage being the variability in Rf values.

Two dimensional chromatography : A margin of 1.5-2 inches is made on both sides and the material under test is spotted at the junction of these margins. The paper is irrigated in the first solvent

for 18 hours, air dried and then run at right angle to the first run in the second solvent. The draw back of this technique is that only one sample can be separated in each run and the advantage is of good resolution.

Preparation of TLC plates : Thirty grams silica gel G and 55 ml of water are stirred and 5 square plates (20 X 20 cm) are coated to a thickness of 0. 3 mm. The plates are allowed to dry at room temperature, the direction of coating is noted and preserved in a desiccator. Just prior to use, the plate is heated at 90 C for 1 hr.

Development of TLC plate : The mixture to be analysed is spotted about 1 inch from the bottom of the plate and the lower edge of the plate is dipped into a suitable solvent in a closed chamber such that the spot is above the solvent level. The solvent rises by capillary action as in paper chromatography and the mixture is resolved into discrete spots. When the solvent front approaches the top which usually takes 30-60 min, the plate is dried and the position of the separated components are located by spraying with a suitable indicator.

b. Column chromatography : Column or classical liquid chromatography has been extensively used in the biomedical field for preparative purposes. Column chromatography is useful in quantitative analysis and in sample purification. Glass columns (the dimensions are dictated by the amount of the sample) filled with the stationary phase (adsorbant, ion exchange or affinity media) are usually used. The sample is applied as a narrow zone at the top of the column and the mobile phase is passed through the column normally under atmospheric pressure. Either an isocratic(solvents in a fixed ratio)or a gradient (where solvent composition is altered in a particular manner-linear, concave, convex or stepwise) elution mode may be used depending on the type of separation aimed at. The eluate is collected in fractions of small volumes for later analysis. Various types of chromatographic media are available for column chromatography and any mode (adsorption, partition, ion exchange, gelpermeation or affinity) may be used.

c. Gel filtration chromatography : One of the most useful and powerful tools of separating proteins from each other on the basis of size is molecular exclusion chromatography, also known as gel filtration or molecular sieve chromatography. It differs from ion exchange chromatography, which separates solutes on the basis of their electric charges and acid-base properties. In molecular exclusion chromatography the mixture of proteins, dissolved in a suitable buffer, is allowed to flow by gravity down a column packed with beads of an inert, highly hydrated polymeric material that has previously been washed and equilibrated with the buffer. Common column materials are Sephadex, Agarose (the commercial name of a polysaccharide derivative) and Bio-gel (a commercial polyacrylamide derivative) all of which can be prepared with different degrees of internal porosity. In the column, proteins of different molecular size penetrate into the internal pores of the beads to different degrees and thus travel down the column at differnt rates. Very large protein molecules cannot enter the pores of the beads, they are excluded and thus remain in the exclusion volume of the column, deflned as the volume of the aqueous phase outside the beads. On the other hand, small proteins are retarded by tha column while large proteins pass through rapidly. Proteins of intermediate size will be excluded from the beads to a degree that depends on their size, hence the term exclusion chromatography. From measurements of the protein concentration in small fractions of the eluate an elution curve can be constructed.

Molecular exclusion chromatography can also be used to separate mixtures of other kinds of macromolecules, as well as very large biostructures, eg. viruses, ribosomes, cell nuclei and bacteria by

using gels with different degrees of internal porosity. The resolving power of molecular exclusion chromatography is now widely used as a way of determining the molecular weight of proteins.

d. Ion exchange chromatography and automatic amino acid analysis : The technique of amino acid analysis by ion exchange chromatography plays a central role in the investigation of amino acid composition of proteins, the determination of free amino acids in physiological fluids.

Ion exchange resins may be defined as polyvalent materials that are insoluble in an aqueous medium and contain functional groups that are capable of exchanging ions from the surrounding medium. Synthetic resins based on a polystyrene matrix are most commonly employed. The principle of separation depends on the differential affinities of various amino acids for the functional group of ion exchange resins.

The amino acid mixture is passed through a column packed with lhe resin and the amino acids eluted by buffers of increasing pH through the column. The amino acids move down the column as separate bands. The effluent containing individual amino acids is then allowed to react with ninhydrin reagent tc form a coloured complex. The intensity of this coloured complex is continuously monitored as it passes through the flow cell of an absorptiometer linked to an electronic recorder. The amino acids are detected as peaks with areas proportional to the respective amounts present in the mixture.

e. Affinity chromatography: Affinity chromatography exploits the abiliıy of enzymes and some other proteins to bind substrates, coenzymes, inhibitors, activators and other ligands specifically and reversibly. The concept is realised by covalently binding the ligand to an insoluble matrix and packing the support into a chromatographic column. In principle only enzymes or proteins which display appreciable affinity for the immobilised ligand will be retained on such a colum, whilst others displaying little or no affinity will pass through unretarded. The specifically adsorbed protein can then subsequently be released by altering the mobile phase to favour dissociation.

f. Gas liquid chromatography (GLC) : Gas liquid chromatography is the method of choice for rapidly and accurately analysing volatile substances. In essence, the volatile material is injected into a column containing a liquid adsorbent (stationary phase) supported on an inert solid. The basis for the separation of the component of volatile mixture is the diffrence in the partition coefficient of the components, as they are carried through the column by an inert gas (mobile phase) such as nitrogen, helium or argon etc.

The resolved compounds are carried over to the detector which presents an electrical signal to the recorder which in turn displays the separated components of the mixture as a series of peaks. These peaks are, detector response varsus time. The area under these peaks is proportional to the amount of substance passing through the detector.

Retention time is the time interval measured from the time of injection of the sample to the time when peak appears. Unknown samples are identified based on the retention time of known standard samples. GLC has been extensively used for the estimation of fatty acids, bile acids, steroids, hormones, sugars and alkaloids.

g. High pressure liquid chromatography (HPLC) : HPLC is one of the major methods of analysis of organic compounds. It offers a high degree of analytical selectivity in addition to the great speed of separation. Its versatality and sensitivity are equalled only by gas liquid chromatography and it has an edge over the latter in that its scope is not limited by sample volatility or thermal stability.

The technique of HPLC involves the separation of the various constituents in a sample mixture followed by their individual detection and quantitation. All chromatogrphic modes (adsorption, partition, ion exchange and molecular sieving) are possible. High separation efficiency is achieved by optimisation of column parameters, particularly the particle size of the column packing. Particles of size 5-10μm are commonly employed (as against the 100μm particles used in classical liquid chromatography). This has necessitated the use of high pressures to obtain adequate flow rates of mobile phase. Detection system forms part of HPLC, ultraviolet, fluorescence and refractive index detectors are most commonly employed for the purpose.

5. ELECTROPHORESIS

Electrophoresis is the migration of particles under the influence of a direct electric current. To apply this technique, two simple requirements must be met. Firstly, the particles to be studied must either be charged or be capable of accepting a charge. Many biological compounds like proteins, amino acids, nucleotides and phosphosugars meet this requirement. Secondly, the medium in which the electrophoresis is performed must be capable of carrying an electric current.

a. Paper electrophoresis : Samples are applied to the centre of the support, ie. filter paper, which is moistened with buffer of appropriate pH and connected to buffer reservoir by paper wicks. When current is applied molecules with a net negative charge at the selected pH migrate toward the anode and those with a net positive charge toward the cathode. For molecules with identical charge the lower molecular weight material migrates farther. Net charge is, however, an important factor in determining separation. For visualisation the dried electrophorogram is sprayed with a coloring reagent (like ninhydrin for amino acids) or exposed to UV light (nucleotides).

b. High voltage electrophoresis (HVE): High voltage electrophoresis is generally understood to imply a potential gradient of atleast 50 volts/cm. But higher potential gradients like 100 volts/cm give optimum separation. Though the principles of ordinary electrophoresis and HVE are same, HVE has some advantages such as,

(i) It can separate particles with almost similar charges including disterio isomers.

(ii) Time of electrophoretic run is considerably reduced, so that diffusion is minimised and sharp discrete spots or bands are obtained.

However, due to the high voltage employed there is often large amounts of heat generated. To dissipate this an efficient cooling system is required. In general, there are two types of HVE apparatus in common use. They are (a) using an organic liquid for cooling and (b) using cooling plates.

Using either type of the apparatus, one can achieve sharp discrete separation of compounds which differ very slightly in their charges from one another.

c. Gel electrophoresis : Gel electrophoresis is an adaptation of electrophoresis. Instead of filter paper, a gel medium is used containing a hydrophobic medium as internal phase and an electrolyte buffer as the external or solvent phase. In gel electrophoresis there are two factors which tend to separate protein or nucleic acid fractions. They are (i) the application of an electric current which produces separation on the basis of charge density (rate of migration in the electric field) and (ii) molecular

sieving (separation based on pore size of the material through which the components of a mixture migrate). Thus an increase in concentration of the solid phase in the gel will result in a decrease in the pore size and greater retention of large molecules.

Three types of gels are used for protein electrophoresis, (i) agar gel, most suitable for electrophoresis of protein containing immune factors, their transparency permits recognition of antigen-anitibody complexes; (ii) starch gel, acts more on the basis of molecular sieving whereas, (iii) acrylamide gel is most useful when both molecular sieving and electrophoretic separations are desired. After separation has taken place the gels are stained to locate the bands and the excess dye removed by destaining of gels.

6. RADIOISOTOPE METHODOLOGY

Radioisotopes have proved as an invaluable tool in biological research. The unusual utility of these radioisotopes are, they can be quantified and located even after they have intermixed with large number of stable atoms of the same element.

Elements of nuclear physics : The matter is composed of atoms, they form the basic building blocks and are extremely small. An atom consists of a nucleus surrounded by electrons. Since the atom as a whole is electrically neutral, the nucleus must carry as many positive charges as there are electrons in the electron cloud to compensate for their negative charges. The nucleus is formed by protons and neutrons, Few terms used in discussing the subatomic particles are defined as follows :

Protons	(Z)	:	are positively charged particles with a mass of unity.
Neutrons	(N)	:	are uncharged particles with a mass of unity.
Electrons	(E)	:	negatively charged particles with negligible mass
Atomic number	(Z)	:	the number of protons.
Atomic mass (Mass number)	(A)	:	the total number (mass of protons and neutrons) (Z+N),

Only certain combinations of protons and neutrons make the atoms (nuclei) stable. These are known as stable isotopes. Nuclei become unstable when they have unstable configurations of protons and neutrons known as radioactive isotopes (N/Z ratio is high or low). Unstable nuclei achieve stability by means of the process of radioactivity. This process involves a change in the number of protons and neutrons in the nucleus or only the release of excess energy from the nucleus.

Isotopes : Atoms of the same element with different mass number. Stable isotope eg. $^{2}_{1}H$ (deuterium), radioactive isotope eg: $^{3}_{1}H$ (Tritium),

Isobars : Atoms having the same mass number but different atomic number. eg. $^{32}_{15}P$ and $^{32}_{16}S$

Isotones : Atoms having the same number of neutrons. eg. $^{2}_{1}H$ and $^{3}_{2}He$

Modes of decay : There are 3 modes of radioactive decay. They are - α, β and γ decay. In biological research only isotopes emitting β and γ decay are used.

a. Interaction of radiation with matter : Positively charged α – particles and negatively charged β-particies are slowed down as they pass through matter, their energy being used to create ions (ionisation) and excited atoms in the material through which they are passing. Gamma radiation interacts with matter in a rather different way. It cannot be slowed down in matter since it consists of electromagnetic radiation which travels with the velocity of light. Gamma radiation like X-radiation is absorbed by three main processes, (i) photoelectric effect, (ii) compton effect and (iii) pair production. These three processes produce secondary electrons which behave like β particles. Various effects arising from the interaction of β and γ radiation with matter can be used for detection and measurement of radioactivity.

b. Physical properties of a radionuclide : The physical properties of a radioactive nuclide determine its usefulness as a tracer. The three most important properties are half life ($t_{\frac{1}{2}}$), mode of decay (α, β or γ) and decay energy.

Half life : The time by which one half of the active atoms of the nuclide have disintegrated.

$t_{\frac{1}{2}} = \frac{0.693}{\lambda}$, where λ = disintegration constant.

The half life period is a natural constant for each isotope. If the half life of a nuclide is very short, any compound labelled with it will be difficult to prepare, use and measure within the time of decay. The mode and energy of decay determine how the nuclide will be measured.

c. Units of radioactivity, Curie (Ci) : Curie is the amount of any isotope which undergoes 3.7×10^{10} disintegrations per second (dps). One curie is equal to the activity of 1 g of pure radium.

The unit of radioactivity in the International system of units (SI) is the Becquerel (Bq).

1 Bq = 1 nuclear transformation/second, therefore,

1 Ci = 3.7×10^{10} dps = 2.2×10^{12} dpm = 37 Giga Bq (GBq)

1 mCi = 3.7×10^{7} dps = 2.2×10^{9} dpm = 37 Mega Bq (MBq)

1 μCi = 3.7×10^{4} dps = 2.2×10^{6} dpm = 37 Kilo Bq (KBq)

1 GBq = 27.027 mCi

1 MBq = 27.027 μCi

1 KBq = 27.027 nCi

Specific Activity (Activity per unit mass) : Because of the small chemical scale on which the labelled compounds are prepared and the small weights and activities normally used in tracer investigation the specific activities are expressed on a millimolar basis (Ci/mmol or mCi/mmol or μCi/mmol).

$$\text{mCi/mmol} = \frac{\text{Total activity (mCi)}}{\text{Mass (mg)}} \times \text{Mol. wt.}$$

Carrier free (Cf) signifies a solution in which all atoms of a particular element are radioactive. Radioactive concentration is normally given as an activity per unit volume (eg. 1 mCi/ml).

Uniform labelling (U): The symbol (U) denotes labelling which is distributed with statistical uniformity over all the atoms.

Specific labelling : This is labelling of radioactivity confined to the indicated positions, eg. Glucose-1-^{14}C or L [Methyl-^{14}C]-methionine.

d. Radiation detection and measurement : Any radiation measuring device will have two components, a detector and a scaler (counter).

Ionisation detectors : In ionisation detectors, energy rich particles of radioactive radiation, transform the molecules of the gas of the detector with which they collide into ions. These charged particles migrate in a high tension field creating a current flow of an intensity proportional to the number of gas ions. The Geiger-Muller counters widely used are based on this principle.

The two types of Geiger-Muller counters widely used are, (i) end window counter and (ii) gas flow counter (windowless counter). The end window counter has a thin window of mica which allows β particles of most energies to enter the counter. In windowless counters the source is inserted into the counting chamber eliminating window absorption and improving geometry. These counters are provided with a continuous flow of an inert gas. The γ-radiation connot be counted efficiently by the ionisation detectors.

Scintillation counters : When radiation interacts with certain substances called fluors (phosphors), a small flash of visible light (a scintillation) is produced. This process constitutes the basis for the operation of all scintillation detectors. The basic scintillation system consists of a scintillation detector, a high voltage supply, a preamplifier and a scaler. There are two types of scintillation counters that are widely used in biological research. They are (i) crystal counters and (ii) liquid scintillation counters.

Crystal counters employ sodium iodide crystal as scintillator. For gamma scintillators, single large crystal of sodium iodide containing a trace of thallium iodide as an activator is alomost exclusively used. Small volumes of radioactivity can be counted efficiently in a well type scintillation counter. This is a cylindrical crystal with a hole cut into one end. The sample in a test tube is inserted into this hole. The geometry is highly favourable, because all the gamma rays emitted by the sample will traverse the crystal. One advantage of gamma ray detectors is that the samples can be counted directly (eg. urine, blood) with out any processing of the samples. Gamma rays are detected at high efficiency with the crystal counters than with Geiger-Muller counters. The beta rays are not detected by these ctystal counters.

Scintillation counters which employ a crystal scintillator for the detection of radiation have a very limited usefulness for the detection of weak beta particles. In liquid scintillation counting, a solution of a fluorescent substance dissolved in aromatic solvents replaces the crystal scintillator. This solution is called as liquid scintillator. The radioactive substance is dissolved directly or suspended, if insoluble in the liquid scintillator. Beta particles emitted, as the radioactive atoms decay, interact with the liquid scintillator to produce very small flash of light. These flashes of light are then detected by a photomultiplier tube.

The most commonly used fluorescent substances and solvent system consists of PPO (2,5-diphenyl oxazole), POPOP (2, 2'-paraphenylene bis 5-phenyloxazole) and toluene. The 'primary fluor' is PPO and this is the substance which converts the β particle energy to light energy. The 'secondary fluor' is POPOP and is used to shift the wavelength of the light emitted by the primary fluor to a longer wavelength to which the photomultiplier tube is more sensitive.

Liquid scintillation counters are used for counting ^{14}C and ^{3}H etc., which emit beta rays. The

main advantage of this counter is the facility for simultaneous determination in a mixture of ^{14}C and ^{3}H, ^{55}Fe and ^{59}Fe etc.

Counting efficiency : Though the emission of radiation is isotropic, the radiation measuring instruments may or may not detect and record all the disintegrations of a radioisotope. This depends upon the energy of radiation, the geometry between the sample and the detector and other factors like self absorption, scattering and quenching etc. The disintegrations recorded by the scaler are known as the counts or counts per minute (cpm). The efficiency of the detector is obtained from the formula:

$$\text{percent efficiency} = \frac{\text{cpm}}{\text{dpm}} \times 100.$$

Backgorund : All radioactivity measuring equipments are subject to a background count rate, arising from external γ and cosmic rays. In order to derive the count rate from the sample alone it is necessary to measure the total count rate from sample and subtract the background count rate observed in the absence of the sample (ie. sample count – background count).

To get a reliable count of a sample the sample has to be counted for a specified time. This time can be obtained from the formula :

$$T = \left\{ \sqrt{\frac{B}{D}} + \sqrt{\frac{B}{D^2} + \frac{1}{D}} \right\}^2 / C^2,$$ where T is the time in min required to get a reliable count, B is the background of the instrument (cpm), D is the cpm of the sample and C is the confidence limit at 5, 1 or 0.1% where C is equal to 0.5, 0.01 or 0.001 respectively.

Quenching is a processs which results in a decrease in the intensity of the flashes of light produced in the liquid scinitillator by the β particles. There are different methods to assess the quenching of a given sample. The internal standard method of determining the counting effciency of quenching consists of counting the sample, adding a radioactive standard and recounting. The latest models of the liquid scintillation counters have the facility to assess the quenching (channel ratio and auto external standard methods) of the sample.

e. Safe handling of radioisotopes: Radioisotopes are classed among the most noxious substances because they may constitute health hazard even when used in trace amounts. Exposure to ionising radiation can give rise to lesions both in the exposed individual and in his descendants.

The health hazard arises in the following ways (i) from external sources emitting radiations, (ii) by accidental ingestion by swallowing or by inhalation and (iii) by contamination of the person (skin, clothing or hair).

Rigorous cleanliness avoids contamination of both apparatus and person. Some general advices are given below :

(i) Segregate work with radioactive substances from other work, (ii) use the minimum quantity of radioactivity needed for the investigation and always work over a suitable spill tray, (iii) do not eat, drink or smoke in an area where radioisotopes are handled, (iv) in addition to overall, wear surgical gloves as normal practice and use forceps to handle the materials, (v) never pipette radioactive solutions by mouth, (vi) segregate apparatus used for high activity work from that used for low activity work. (vii) disposal of

the radioactive waste can be achieved suitably depending upon the activity and the half life of isotope used and (viii) use personal monitoring devices.

7. SATURATION ANALYSIS

The distinguishing concept of saturation analysis is that, by allowing the substance to be measured (P) to react with a specific binding reagent (Q) of limited capacity, substance P by saturating Q is partitioned into two moieties (bound and free) such that the ratio of the two varies as a fuction of the total amount of P present.

In order to utilise this analytical principle, means must be devised for separating or identifying the two compartments and subsequently, for quantifying the distribution of P. A variety of ways of achieving this objective may be proposed, although, in practice, only one approach has been substantially exploited in the past. This approach relies on the addition of radioactive material (usually, though not necessarily identical to P) to the system. Following the physical separation of the bound (PQ) and residual free P moieties, the partition of radioactivity between them is observed. Thus, with increasing amounts of unlabeled P present, the amount of bound P decreases. The use of radioactive material is not the fundamental feature of saturation analysis, it may be substituted by other markers such as enzyme (enyme immunoassay or ELISA) or viral particle (viro immunoassay).

If the competition between the labeled and unlabeled ligands is allowed to occur simultaneously until mass equilibrium is reached, the technique is reffered to as 'equilibrium saturation'. In order to eliminate the simultaneous competition, the labeled ligand may be added after some delay and the reaction is stopped before equilibrium has been reached. This technique is referred to as 'nonequilibrium analysis' or 'sequential saturation'. In practice, equilibrium assays have been used by most investigators. Sequential saturation has been used only when problems related to sensitivity or larger differences of labeled and unlabeled ligands for the binder have to be overcome.

There are different methods for quantitating the free antigen or the antigen - antibody complex. The most commonly used immunoassays are(a) radioimmunoassay, (b) enzyme linked immunoassay and (c) radioimmunodiffusion technique.

a. Radioimmunoassay (RIA): The basic principle of radioimmunoassay is saturation analysis. The components of such saturation analysis techniques include, (i) a radioisotope labeled antigen or ligand, (a ligand is a small molecule that can be bound by a larger molecule), (ii) standard antigen or ligand, (iii) specific antibody or binding protein.

The technique involves (a) reaction between the labeled antigen (ligand) with the specific antibody (binding protein), (b) competition of the standard nonlabeled antigen for the same limited number of binding sites on the antibody resulting in a dose dependent reduction in the amount of labeled antigen bound. By using varying concentrations of the nonlabeled antigen, an unknown can be measured.

b. Enzyme linked immunoassay (ELISA): ELISA is based on the reaction between antibodies and antigen which are linked to a solid phase. By addition of a specific anti–immunoglobulin conjugated to an enzyme, the antibodies that have reacted with the antigen become detectable. This is done by adding the specific substrate for the enzyme.

The steps involved are, (i) an antigen or antibody is linked to a carrier surface usually the inside of the wells of a particular micro ELISA plate; this carrier surface captures the corresponding antibody/ antigen from a test solution, (ii) an enzyme labeled antiglobulin attaches to the antigen/antibody complex, (iii) the antigen/antibody complex is detected by the enzyme label which changes colourless substrate to a coloured compound and (iv) the OD of the final colour is directly proportional to the amount of unknown antibody/antigen in the original test solution. The result may be read as either an end point titration value or as quantitative OD mesurement.

c. Radioimmunodiffusion technique: This technique is generally employed for the quantitation of immunoglobulins, complement compound C_3, retinol binding protein (RBP) or any other antigen.

The protein antigen diffuses radially from a point of application into an antibody containing gel. A circular precipitate results at the point of equivalence. With constant or uniform antibody concentration and constant gel thickness, the circular area formed as precipitation ring is proportional to the concentration of antigen.

8. MICROBIOLOGICAL ASSAYS

Microbiological methods are based on the observation that certain microorganisms require specific nutrients for growth. Using a basal medium complete in all respects except for the nutrient under test, growth responses of the organism are compared quantitatively in standard and unknown solutions. Either the acid or the turbidity produced by the organism is meaused to determine the extent of growth and thereby the amount of nutrient in the test solution.

Microbiological assays have been widely used for the determination of vitamins and amino acids.

Preparation and maintenance of stock cultures: Stock culture agar tubes are prepared and inoculated with the pure culture (appropriate organism to be used for the different assays). The tubes are then incubated for 16 – 24 hr at 37°C and stored at 2 – 4°C. The cultures are transferred into new agar tubes every fortnight.

Preparation of stock culture tubes :

Difco peptone	..	5 g
Difco yeast extract	..	1 g
Glucose anhydrous	..	10 g
Sodium acetate $3H_2O$ (or)	..	17 g
Sodium acetate anhydrous	..	10 g
*Salt A	..	2.5 ml
*Salt B	..	2.5 ml

*Salt A : K_2HPO_4 – 25 g, KH_2PO_4 – 25 g. Dissolved in water and made upto 250 ml.
*Salt B : $MgSO_4.7H_2O$ – 10 g, NaCl – 0.5 g, $FeSO_4.7H_2O$ – 0.5 g, $MnSO_4.H_2O$ — 0.5 g : Dissolved in 250 ml water to which are added a few drops of conc. HCl to obtain a clear solution.

The above ingredients are dissolved in 200 ml distilled water, the pH adjusted to 6.8 and the volume made upto 250 ml. Agar 7.5 g is dissolved in 250 ml of distilled water by heating and mixed

with the above solution. While the solution is still hot, 10 ml of this well mixed solution is delivered into test tubes which are then plugged with cotton and autoclaved for 15 min at 12 lb pressure. After cooling to room temperature the tubes are stored at 2 – 4°C and used for maintaining stock cultures of *Lactobacillus casei, Streptococcus faecalis, Lactobacillus arabinosus, Leuconostoc mesenteroides* or *Paediococcus cerevisiae.*

Preparation of inoculum broth The inoculum broth is prepared in the same way as the culture medium excepting that instead of the agar solution more of distilled water is used (after adjusting the pH to 6.8) to make up the volume to 500 ml. Ten ml of this solution is delivered into test tubes which are then plugged with cotton, autoclaved as above, cooled to room temperature and stored at 2 – 4°C.

Preparation of inoculum : The day prior to use, cells from the stock culture are transferred to the inoculum broth tube and incubated for 16-18 hr at 37°C. A centrifuge tube plugged with cotton, a syringe, a needle and some saline (0.9%NaCl) in a conical flask, all are sterilised by autoclaving for 15 min at 15 lb pressure. The cells from the inoculum tubes are transferred to the centrifuge tube and centrifuged, the supernatant decanted off and the cells resuspended in sterile saline and centrifuged. The process is repeated 2 to 3 times. The washed cells are again made upto a suspension using the saline. The resuspended cells are taken in the sterile syringe and the assay tubes are inoculated with one drop of the inoculum.

Preparation of standard tubes: The stock standard solution is diluted (according to the concentration) to give the working standard solution. The latter is prepared fresh each day. To duplicate assay tubes are added aliquots of the working standard solution at different levels. Sufficient water is added to bring the volume in each tube to a known level (5.0 ml in case of vitamins and 1.0 ml for amino acids). To each of these is then added a known volume of the medium solution (5.0 ml for vitamins and 1.0 ml for amino acids).

Preparation of samples: The test sample is weighed and treated for extraction of vitamins or hydrolysed to obtain amino acids (details of procedure given under individual assays). The pH is adjusted and the solution diluted to the proper concentration.

Preparation of assay tubes: To duplicate tubes are added aliquots of the test solution and the volume made up with water. This is followed by the addition of the basal medium. The tubes are covered with lint cotton and brown paper or with caps, autoclaved and cooled to room temperature. Each tube is then aseptically inoculated with one drop of the inoculum, the tubes are shaken well and incubated at 37°C for 24 – 72 hr. At the end of the incubation period the assay tubes may be read turbidimetrically in a colorimeter at 660 nm using an uninoculated blank. An incubation period of 18-24 hr is enough for assaying turbidimetrically. For determining titrimetrically, an incubation period of 72 hr is needed.

Titration : The contents are transferred quantitatively to a 100 ml conical flask, rinsing the tubes with distilled water twice. About 0.2 ml of 0.1% bromothymol blue indicator is added and the solution titrated against standard NaOH to an end point of greenish blue colour around a pH of 6 8.

Calculation : A standard curve for the assay is drawn by plotting the volume of standard NaOH (if turbidimetry, the optical density readings) on the Y-axis against concentration of the vitamin or amino acid per tube in the standard series on the X - axis. The vitamin or amino acid content of the tubes in the unknown series is determined by interpolation of the titer values or colorimeter readings on the standard curve. The average for 1 ml of test solution is calculated from values obtained from not less than 3 tubes which do not vary by more than 10% of the average. The vitamin or amino acid content of the test solution is calculated using the following relationship :

$$\mu g \text{ of test nutrient/g sample} = \frac{\text{Average } \mu g \text{ per ml X volume X dilution factor}}{\text{Weight of the sample.}}$$

9. CENTRIFUGATION

When a particle revolves around an axis a force is developed and acts away from the axis of rotation. This force is called centrifugal force. A particle suspended in a medium takes some time to sediment, which depends upon the size or molecular weight of the particle, the viscosity or density of the medium, under normal gravitational force. To speed up the process of sedimentation the external field of force is allowed to act on the particle which depends also on the distance of the particle from the axis of rotation apart from the other two factors mentioned earlier.

Centrifugation is generally classified into two types (i) analytical and (ii) preparative. Analytical centrifugation is useful in studying the physical properties of the substance while preparative centrifugation is useful where the supernatant or sediment can be used for further precessing. Ultra-centrifugation is a process of separation of particles whose molecular weights are very low and which do not get sedimented under fields of force applied in ordinary centrifugation. To achieve ultraspeeds, extra protection is offered to the motor by the application of refrigeration and vacuum.

Practical aspects:

1. Separation of particles by size
2. Differential pelleting
3. Analytical centrifugation
4. Rate zonal centrifugation
5. Separation of particles by density.

Uses:

1. Separation of serum proteins
2. Separation of lipo proteins
3. Separation of living cells and organelles
4. Determination of molecular weight
5. Purity of colloids.

Precautions:

1. Balancing and loading of tubes and buckets must be accurate and proper.
2. The specific gravity of medium should be less than the specific gravity of particle under sedimentation. For specific gravities of media of more than 1.2 g/ml the maximum allowed speed must be derated using the formula.

$$\text{Derated speed} = \sqrt{\frac{1.2}{\text{Specific gravity of media}}} \times \text{maximum allowed speed.}$$

3. If steel or other tubes are used instead of poly carbonate or plastic tubes, then the maximum speed must be derated.

Derated speed $= \sqrt{\frac{A}{B}}$ x maximum allowed speed, where

A = weight of plastic tube and contents.

B = weight of steel or other tube and content.

4. Under no circumstances the maximum speed should be exceeded.

The relative centrifulgal forces RCF (g) $= \frac{\omega^2 r}{980}$

ω = Angular velocity $= \frac{2\pi}{60}$ x rpm

r = Particle distance from the axis or radius of rotor in cm.

$g = 1118 \times r \times (rpm)^2 \times 10^{-8}$

Density gradient centrifugation: Density gradient or zonal centrifugation is widely used and is a versatile procedure for separating not only proteins and other macromolecules but also organelles and viruses. In the most common procedure a continuous density gradient of rucrose is first prepared in a plastic centrifuge tube by a device that mixes concentrated sucrose solution and water in decreasing ratio as the tube is filled, so that the density of the medium is greatest at the bottom of the tube. The mixture of macromolecules to be resolved is layered on top of the gradient. Centrifugation of the tube in a horizontal position in a rotor at a high speed causes each type of macromolecule to sediment down the density gradient at its own rate, determined primarily by its particle weight and by density and shape in the form of sepearate bands or zones. Usually, centrifugation is stopped before equilibrium is reached. The positions of the protein bands can be located optically or by draining off the contents of the tube carefully through a pinhole in the bottom and analyzing succssive small samples. Alternatively, the plastic tube can be frozen solid and then cut into thin slices for analysis.

10. DIALYSIS AND ULTRAFILTRATION

In biological fluids sometimes macromolecules need to be separated from micromolecules, This can be achieved by number of procedures. The most commonly used methods are dialysis and ultrafiltration.

Dialysis: The main purpose of dialysis in biological research is the removal of small molecular weight constituents from biological fluids. This is achieved by dialysing the desired material in a cellulose tubing with a pore size of 40–80 Å (4-8 nm) diameter which allows the passage of compounds with molecular weight less than 10,000. The following procedure can be conveniently adopted.

Cut the length of the dialysis tubing depending upon the volume to be processed. Open the sides of the tubing with a flow of glass distilled water and transfer it to a boiling water bath. After this processing the tubing should never be allowed to dry as this will cause distortion of pore size. Make a bag by tying a knot at one end of the tubing. Transfer the solution to be dialysed into this bag. Tie another knot at the top of the bag. The bag is then put in a beaker containing the solution against which dialysis is to be carried out. In general one volume of desired solution is dialysed against 50 to 100 volumes buffer. The total period of dialysis could range from 8-24 hr. The efficiency of dialysis could be increased by stirring and by changing the dialysate solution at frequent intervals.

Ultrafiltration : Ultrafiltration is a technique of separating high molecular weight compounds like proteins, nucleic acids etc., from small molecular weight compounds. Similar to the technique of dialysis this method also utilises a semipermeable membrane to retain the large molecular weight compounds and allows the water or small molecular weight compounds to pass through. The technique consists of using a semipermeable filter supported on a grid, loading the sample and applying either centrifugal forces or high pressures to separate the small molecular weight compounds. The typical membranes used are made of cellophane.

11. LYOPHILISATION AND FLASH EVAPORATION

Lyophilisation : Lyophilisation or freeze drying provides a reliable method of drying thermolabile material. Dehydration of the specimen is carried cut at a low temperature under reduced pressure. Under these conditions chemical and physiological properties of the specimen are retained.

Freeze drying is the removal of water by sublimation at low temperature from a specimen after it has been frozen. The solution can be frozen to the walls of a round bottomed flask, by rotating the flask in an alcohol bath at -20°C. The flask is connected to one of the parts of multiport chamber provided on the lyophiliser through a quick seal valve and the system is closed to the atmosphere and evacuated by a mechanical pump, the condenser coils having been precooled to about -40°C to ensure efficient condensation of water vapours on the condensing coils. When the pressure inside the system drops to 0.3 mm of Hg, a small amount of heat may be applied to the sample initially to enhance the rate of lyophilisation. As the stage is approached when all the free ice has sublimed as indicated by a fall in pressure and a rise in temperature of the sample, the application of heat may be stopped. The primary drying is then complete. Residual moisture may be removed using a secondary drier such as P_2O_5.

Flash evaporation : This provides a means of rapid evaporation by rotating a flask, containing the solution to be concentrated in a thermostatically controlled heating bath. Surface area of the solution in a half filled flask is approximately tripled by production of a thin film on the inner surface. The evaporation is greatly accelerated at a temperature much below the normal boiling point of the solvent, since the evaporation is carried out under vacuum. The rotation of the evaporator eliminates bumping and foming. The vapours of the solvent are condensed and collected in a condenser flask precooled by water.

12. MEASUREMENT OF pH

Water in solution has the capacity to dissociate into H^+ and OH^- ions. When substances are dissolved in water, depending on their degree of dissociation and their chemical nature, can accept H^+ of water and makes it either 'acidic' or 'basic'. The concept of acids and bases can be defined, as a substance which can donate or accept protons, respectively.

All acids or bases do not confer the same amount of acidity or alkalinity to the solutions. For example, a certain volume of 0.1 N sodium hydroxide can effectively neutralise the same volume of 0.1 N HCl or 0.1 N acetic acid. However, in terms of acidity 0.1 N HCl is about 71 times more acidic than 0.1 N acetic acid. This is because all the hydrogen of HCl exists as H^+ whereas only 1.3% of acetic acid hydrogen remains as H^+. Since this change in acidity or alkalinity alters the characteristics of the substances present in the solution like proteins etc., it is essential to quantitate this phenomenon.

The quantification of this was achieved by studying the dissociation of water. The product of the concentration H^+ and OH^- in pure water at 22°C is found to be $1X10^{-14}$. Since water is neutral in character, then the concentration of $[H^+] = [OH^-] = 10^{-7}$ moles per liter. The ionic product of water is written as follows:

$$[H^+]\ [OH^-] = K\ [HOH] = K_w = 1X10^{-14}.$$

Then it follows that addition of H^+ by acids or removal of it by bases will lead simultaneously to a change in OH^- to maintain the ionic product of water at $1X10^{-14}$. For example, the concentration of hydrogen in 0.01 N HCl will be $1X10^{-2}$ since all hydrogen ions in HCl will be dissociated. Then the concentration of OH^- will become $\frac{10^{-14}}{10^{-2}} = 10^{-12}$. On the other hand in 0.01 N acetic acid the concentration of H^+ is $4.47X10^{-4}$. Now one can understand why the acidity of acetic acid (0.1 N) is less than that of 0.1 N HCl since in the former H^+ concentration is low.

To avoid these cumbersome numbers in the expression of H^+ concentration, Sorenson in 1909 developed a scale by expressing the same values in a logarithmic manner. Accordingly pH is defined as the negative logarithm of $[H^+]$, $pH = \log_{10} [H^+]$

Then the pH of 0.01 N HCl becomes $-\log_{10} [10^{-2}] = -(-2) = 2$ and the pH of 0.01N acetic acid $-\log_{10} (4.47X10^{-4}) = 3.35$.

Sorenson's scale of pH extends from 0 to 14 taking the hydrogen ion concentration of 1 M to 10^{-14}M and can cover the range from 1 N HCl to 1 N NaOH. So as the pH decreases the $[H^+]$ increases and vice versa. Eventhough pH below 0 and above 14 are possible, 0 to 14 is the range one generally comes across in practice. For every unit change in pH, there will be 10 times change in the $[H^+]$.

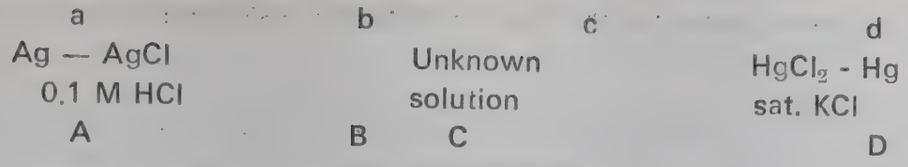
Measurement of pH: The pH of solutions, especially biological specimens is quite critical. The measurement of this parameter has to be accurate and sensitive.

The best and widely used method of pH determination is by measuring the electromotive force (emf) produced by H^+ around an electrode. Here the basic principle is that when two electrodes are kept in two solutions having differing H^+ concentration and the electrodes are connected to a potentiometer and the solutions by a salt bridge, a small potential difference can be observed (emf). If one knows the standard emf produced by a certain concentration of H^+ the emf of other solutions can be measured by comparison. Electromotive force is related to pH by the following equation :

$$pH = \frac{emf}{0.00019837\ T},$$ where T is the absolute temperature.

The hydrogen electrode (platinum with platinum black) with hydrogen gas at one atmospheric pressure and immersed in a 1 N H^+ solution is assigned a potential of zero, under all conditions and is used as the standard reference electrode.

Glass electrode: This method of determining pH is the widely used one at present. The principle according to which the glass electrode functions is simple. It has been found that thin membranes of the proper glass are selectively permeable to H^+ and if a cell is set as follows,



The $[H^+]$ and pH of an unknown solution may be determined. A - represents a silver-silver chloride in half cell containing 0.1 M HCl, C – is the unknown solution to be tested, D is a standard calomel half cell and B is the glass membrane with a definite constant H^+ activity on one side (0.1 M HCl) and a variable H^+ activity on the other (unknown solution). The potential across a glass membrane is a concentration potential due to differences in $[H^+]$.

There are potentials at the boundaries a, c and d, but these are constant for a given electrode and can be denoted as E'c. The value of this may be obtained by using a solution of known pH in C and measuring the potential E. Now the pH of any unknown solution can be obtained by the following formula :

$$pH = \frac{E - E'c}{0.00019837\ T},$$ where E is emf seen in unknown solution, E' c is the emf constant of the electrode and T is the absolute temperature. In actual practice an instrument is calibrated against a series of buffers of known pH. When pH is plotted against E, there should be a smooth curve, practically a straight line between pH values of 1 to 9.

The advantages of a glass electrode are (i) it is applicable to all solutions including those containing oxidising and reducing agents, (ii) it can be used to measure the pH of semisolid material like cheese, (iii) it is ideal for use with biological fluids, (iv) solutions tested by glass electrodes are not contaminated in the process and (v) the accuracy of the glass electrode is ordinarily within a few hundreths of a pH unit.

The disadvantage with glass electrode is that at higher pH beyond 10 it does not function well. Beyond this pH, Na^+ of the glass membrane start getting solubilised and the resultant Na^+ can interfere with H^+ potential and which will not be the same.

13. BALANCES.

Equal arm balance : Consists of a rigid beam having a central knife edge resting on a horizontal bearing plane mounted on a vertical pillar and two arms of equal length. The two ends of the beam carry knife edges resting on bearing planes mounted in stirrups from which the two pans of the balance are hung. A pointer fixed to the centre of the beam and moving over a graduated scale mounted at the bottom of the vertical pillar is used to indicate the equilibrium position of the beam or the rest point of the pointer. When both pans are empty, the beam remains horizontal and the balance is said to be counterpoised. If a sample with a unknown mass is now added to the left hand pan, the pointer will be deflected. Standard weights are then added to the right hand pan till the original position of the pointer is restored. Since the two arms of the balance are equal, the mass of the sample in the left hand pan is equal to the mass of the standard weights added to the right hand pan.

Substitution balance (single pan balance) : This balance has but one pan and two knife edges. The pan and the set of standard weights are both hung from the front knife edge mounted at one end of the beam. The second central knife edge acts as the fulcrum of the beam. A fixed counter weight at

the rear end of the beam balances the load at the front knife edge consisting of the pan and the calibrated weights.

When an unknown weight is placed on the pan, the beam deflects in the direction of the heavier side. To return the system to equilibrium, the corresponding mass of calibrated weight is removed from the front suspension. The mass of calibrated weights so removed gives the mass of the unknown placed on the pan.

14. COLLECTION OF BIOLOGICAL SAMPLES

Time of sample collection : In order to maintain comparable conditions blood samples are usually collected after an overnight fasting between 9.00 - 10.30 AM. The fixed time schedule eliminates the possible effect of diurnal variations.

Collection of blood : There are two sources of blood for laboratory tests - capillary peripheral blood and venous blood. For small quantities of blood, puncture of finger tip or heel is adequate. The part punctured must not be cyanotic or edematous. Skin should be cleansed with alcohol and rubbed dry. Puncture should be made with a quick stroke of lancet sufficiently deep to produce spontaneous flow of blood. The first drop should be discarded before sample is collected. Undue pressure near the puncture site to secure drops of plood should be avoided. For blood smears it is preferable to make smears directly from blood obtained by finger/heel prick immediately. If larger volume of blood is needed venipuncture may be the ideal method. Venipuncture is best made in the antecubital vein but when this fails one of the smaller veins in the back of hand or wrist may be useful.

Haemolysis interferes with many estmations. It can be minimised by,

(a) Using clean dry syringe and glassware

(b) Not using too narrow guage (20 or 21 guage) needle for blood drawing

(c) Drawing blood slowly from the vein

(d) Avoiding a mixture of air resulting in frothing while drawing blood and

(e) Removing the needle and gently allowing blood to trickle down the sides of the tube.

If whole blood or plasma is needed, blood is to be transferred to containers having the anticoagulant in the form of a thin dried film on the inner surface. The tube is rotated thoroughly but gently to dissolve the anticoagulants. The commonly used anticoagulants are listed below.

		Amount (mg/ml of blood)
Heparin	..	0.2
Potassium, ammonium oxalate	...	1.2
Sodium citrate	..	6.0
Sodium fluoride	..	10.0
EDTA	..	1.0

For determination of many constituents of blood, serum can be used instead of plasma. Using plasma instead of serum has the following advantages :

(a) There will not be any delay in separating cells from plasma and this may be important in preventing substances that diffuse from formed elements into plasma.

(b) Greater volume of plasma than serum is obtainable from a given volume of whole blood.

(c) Haemolysis can be avoided if plasma is used.

Clotting of whole blood can be delayed if blood is collected in paraffin coated or siliconised glassware. This technique is used principally in research requiring plasma to be obtained without anticoagulant.

Milk collection: Composition of milk shows considerable diurnal variations. In addition, the composition especially lipid profile is known to show differences depending upon whether estimations were made in fore, mid or hind milk samples. Collection of milk samples at the same time of day usually between 9.00 – 10.30 A. M. will eliminate possible diurnal variations. Atleast 2 hr should have elapsed since last breast feed before samples are collected. Complete emptying of breast using breast pump might be attempted to obviate differences in composition due to any one portion of milk being examined. Often there is considerable difference in volume of milk obtained and it is not possible to be certain that the breast has been completely emptied. In case complete emptying is not possible the first 10 ml of milk collected can be taken as representing fore milk. If the baby is allowed to suckle and after suckling, the remaining few ml of milk is expelled from the breast the sample can be taken as representing hind milk.

Urine collection: Urine examination remains one of the simplest noninvasive and widely used investigative procedures. The results of these investigations, however, may be vitiated if proper care is not taken in obtaining a representative specimen using appropriate collection procedure. For most of the urinalysis procedures, pregnancy diagnostic test etc., early morning midstream specimen is the preferred sample. Midstream specimen obtained after proper precautions to avoid contamination of the sterile container is essential for bacteriological examination of urine. For several metabolic investigations and measurement of some excreted products a 24 hr urinary collection may be essential. If dietary intake and climatic conditions remain unaltered urinary creatinine may serve as a useful though not infallible indication of completeness of 24 hr collection.

15. METABOLIC BALANCE STUDIES

The principle in the study of balance of any nutrient involves estimating the intake and excretion of the nutrient. In an adult if the excretion is equivalent to the intake, the person is said to be in equilibrium with respect to that nutrient. In growing organism and those recovering from stress a positive balance, ie. intake is more than excretion is imperative for the maintenance of normal health. A negative balance of any nutrient is indicated by a higher excretion than the intake. The balance studies are useful to study the adequacy of a diet with respect to various nutrients comprising the diet.

Balance studies are commonly employed in arriving at the requirements of different nutrients. A diet of a particular composition with known concentration of a nutrient is ingested for a long enough period for the stabilisation of the body with that nutrient. At this stage the difference between the amount ingested and excreted indicates whether the diet is in excess, adequate or inadequate with respect to the nutrient under study. By changing the level of intake of the nutrient in the diet one can arrive at a point at which a balance is obtained between intake and excretion. This level of intake of the diet is said to meet the requirement of the body for the nutrient.

Balance studies in humans : Balance studies in humans provide useful information if meticulous care is taken to control the intake and scrupulous methods are employed to estimate the nutrient in the excretory produts. In general, it is necessary to house the subjects of the study in a ward or a special home to have a proper control of their intake and for the careful collection of their excreta. Care has to be taken to quantitate the exact intake of the nutrient, the balance of which has to be determined. Weighed diets are to be cooked for individual subjects and a duplicate cooked meal may be analysed for the content of the nutrient of the study. After stabilisation on the experimental diet which is indicated by the constant daily excretion of the nutrient, the urinary, fecal, sweat and other losses of the nutrient should be carefully measured. The difference between the intake and the sum total of the excretions of the nutrient gives the balance.

Balance studies in experimental animals : While the same principles employed in the human balance studies hold good for animals, the techniques of collection of the excreta require modification and depend on the animal of the study. For rats, mice or guinea pigs and such small animals, metabolic cages may be employed for the collection of urine and feces. The cages have to be designed in such a way that the urine drains out into a bottle kept at the bottom of the cage with appropriate preservative and the fecal matter remains on the bottom mesh of the cage. The success of the balance study in animals depend upon the care that is taken to measure the food intake and the collection of urine and feces without contamination with the food material. The food may be served in a separate compartment attached to the cage such that the food cannot be spilled by the animal into the metabolic cage. By giving a weighed amount of food and knowing the left over, one can know the food intake and thus the intake of the particular nutrient of the study. A filter may be provided to the funnel and the fecal material that may escape along with the urine may be blocked and removed carefully and added to the total fecal excretion. The amount of the nutrient excreted in the feces is estimated. The volume of urine collected in the bottle at the bottom of the cage is measured and the total nutrient excreted in urine is estimated. The difference between the intake and the excretion gives the balance of the nutrient.

16. PHARMACOKINETICS

Pharmacokinetics is the study of the time course of absorption, distribution, metabolism and excretion of drugs and their metabolites in the body. The amount of drugs and their metabolites are measured as a function of time in readily available biological fluids such as blood and urine after the administration of the drugs. Mathematical models are then applied to predict quantitatively these dynamic processes. The data on pharmacokinetic profile of a drug will enable the clinician to design the correct dosage schedule that will maintain a desired pharmacological effect.

To explain the complex biological phenomenon of drug absorption, distribution and excretion, the body has been treated as having different 'compartments' though these compartments lack physiological or anatomical reality. The one compartment model considers the body as a single homogenous unit. The two compartment open model consists of a central compartment which includes blood and vascular organs such as lungs and liver, and a peripheral or tissue compartment consisting of muscle and adipose tissue. For some drugs complicated multicompartment systems are used.

It is useful to consider the case of a drug administered in a single dose, either intravenously or orally to define the meaning and significance of several important pharmacokinetic terms.

Biological half life of a drug ($t_{\frac{1}{2}}$) : The half life of a drug is defined as the time required for the

drug concentration to decrease by 50%. A plot of drug concentrations on a logarithmic scale vs time on a normal scale is linear after the absorption and distribution. The slope of this linear phase provides a measure of the biological half life of the drug. The biological half life of a drug in a given patient is usually an important determinant of the duration of pharmacologic effect and of the optimum dosage regimen.

Elimination rate constant (*K or* β) : Elimination of most drugs from the body follow first order kinetics. The fraction of drug that is eliminated from the body in any given time period is constant and directly proportional to the amount of drug in the body. The elimination rate constant is the sum of the rate constants of a number of individual processes such as renal excretion, biliary excretion and metabolism. The rate constant is calculated from the terminal slope (b) of the plasma concentrations vs time plotted on a semilog paper, since K is expressed in units, hr^{-1}, it is essential to multiply the slope (b) with 2.303, which is the factor to convert in to $\log_{10}$. The biological half life of drug is related to elimination rate constant by the equation; $t_{\frac{1}{2}} = \frac{0.693}{K}$, in this equation 0.693 is just natural logarithm of 2.

Elimination rate constant and half life of the drug should be considered in fixation of dose and dosing interval. Failure to take into consideration the factors which influence these phenomena can lead to severe undermedication or dangerous drug accumulation leading to toxicity.

Bioavailability (Area under the curve; AUC $\int_0^\infty$ cdt) : Bioavailability has been defined as the measurement of both the relative amount of an administered dose that reaches the general circulation and the rate at which this occurs. Bioavailability of oral preparations can vary due to many pharmaceutical and physiological variables. Since most popular mode of drug administration is the oral route, data on bioavailabilty is essential for successful therapy.

Bioavailability of a drug is determined by measuring the area under the plasma concentration vs time curve (AUC) from time zero to infinity after oral and intravenous administration of a drug. The AUC upto the last plasma concentration determined can be measured by the use of planimeter or more systematically by employing trapezoidal rule, in which the area under the curve is divided into a number of trapezoids and the area of each trapezoid is calculated. The sum of areas of all trapezoids yields an estimate of the true AUC. The equation for trapezoidal rule is,

$\int_{t_o}^{t_1} ctdt = \frac{t_1 - t_o}{2} (c_o - c_1)$ where c_o and c_1 are the plasma concentrations at time t_o an t_1 respectively.

AUC from last plasma concentration (ct) to infinity is calculated, by dividing the ct with first order elimination rate constant (K). The bioavailability of an oral dose is then expressed;

$$\text{Bioavailability} = \frac{\text{AUC after oral dose}}{\text{AUC after i. v. dose}} \times 100$$

Apparent volume of distribution (*V or* V_B) : The relationship between the amount of drug in the total body at any given time is expressed as apparent volume of distribution. Despite its units of liters/Kg, the

volume of distribution does not represent a body space, but useful in describing the distribution of drug. Volume of distribution can be calculated by either of the following equations :

$V = \frac{x_o}{c_o}$ where x_o is the intravenous dose administered and c_o is the plasma concentration of drug at time zero, generally obtained by extrapolation of plasma concentration to zero time, and $V = \frac{x_o}{\beta . AUC}$.

The first equation can be employed to calculate volume of distribution only when the drug follows one compartment model. On the other hand, second equation is model independent.

Total body clearance (TBC) : Whereas β represents the complex process of drug distribution, biotransformation and urinary elimination, total body clearance indicates the sum of the individual clearnces of the drug by various organs and tissues of the body. It is calculated by multiplying the apparent volume of distribution (V_B) with first order elimination rate constant (β) after distribution phase, $TBC = V_B . \beta$,

It is shown after derivation that total body clearance is equal to drug clearance from central compartment ($V^C K_{10}$) where Vc and K_{10} are apparent volume of distribution and first order elimination rate constant of central compartment respectively.

Since, total body clearance and renal clearance are often the only drug clearance values determined in man, the difference between these clearances would indicate the ' non renal clearance ' of drug.

Protein binding of drugs : Many drugs, particularly acidic and basic drugs bind to albumin but some basic drugs to globulins. Generally, highly protein bound drugs will have a long biological half life, since bound fraction is not subjected to glomerular filtration and will not reach in adequate concentration at the site of biotransformation. In addition, only unbound fraction of any drug will exert their activity. When more than one drug is administered, due to competition for binding sites, drug interactions can result. Hence, the clinical implications of serum protein binding are quite far reaching. However, it has to be emphasised that only in cases of highly bound agents will binding be important in practical sense.

The relative binding of a drug to components of tissues and body fluids is constant and independdent of drug concentration. This is the basis of apparent volume of distribution which is dependent upon both the amount of drug that is plasma bound and tissue bound.

17. QUALITY CONTROL

Quality control techniques attempt to assess the reliability of a laboratory measurement. If repeated estimations are carried out on the same sample, a scatter in values will be obtained. The term 'precision' pertains to this scatter. Greater the precision, lesser will be the scatter or range of values. The term 'accuracy' is concerned with the relationship of a set of results to the true value. A method may be precise, but it may inherently tend to yield higher or lower values compared to the true value. Quality control permits us to determine the variation and also its accuracy and precision.

Use of internal and/or external standards with each batch of assays and calculating its variance over a period of time can provide an effective check for an ongoing method. For routine serum chemistry measurements, standard quality control sera are available. Alternatively, a laboratory can prepare its own quality control serum for routine use.

Calculation of standard deviation and the coefficient of variation : The standard deviation is a good index of the distribution of a set of results in a quantitative manner and can be used to determine the precision of a test. It is calculated by determining the mean of a set of results ($\bar{X}$), calculating how each result (X) varies from that mean ($\bar{X}$-X) and squaring the difference ($\bar{X}$-X)^2. The sum of all the differences squared is then calculated $\sum(\bar{X}-X)^2$. This value is divided by the number of results observed minus one (n-1). The square root of the resultant value is the standard deviation (SD). The coefficient of variation (CV) is the standard deviation calculated as a percentage of the mean value and is therefore,

$$CV = \frac{SD}{\bar{X}} \times 100$$

There are atleast three stages of quality control. When a new technique is being set up, these stages should be gone through in a step wise manner, solving the problems that arise at each stage. The various stages are as follows:

Stage 1 Optimal conditions variance

Stage 2 Routine conditions variance (known values)

Stage 3 Routine conditions variance (unknown values)

Stage 1, Optimal conditions variance : Using a control material, the laboratory should attempt to obtain as low a variance as possible for each of the determinations in the survey. Such endeavours to obtain a low variance may include some procedures which may not be feasible in day to day work. Laboratories will have both common and individual approaches to obtain this variance. The following list is not necessarily complete for each method, but is meant as a guide to the approach. Ideally, approximately 20 analyses as close together in time as possible should be performed.

(i) Use fresh and carefully checked standards. Standardise close to the stated value.

(ii) Use fresh and carefully checked reagents. Protect them as far as possible from light and temperature changes.

(iii) Use the same sampling pipette.

(iv) Pay careful regard to mixing, ambient temperature fluctuation and extreme light exposure during the analytical process or chemical reaction stage.

(v) Carefully check colorimeter readings.

(vi) Check calculations.

(vii) Use laboratory staff who are experienced.

Calculate the mean and standard deviation for all the results. If any result is excluded the reason should be stated.

Plot the results and see for trends occuring between analyses, if they occur, try to explain them.

Stage 2, Routine conditions variance (known values) : First continue with the control material used for the determination of optimal conditions variance but now try to 'mimic' routine conditions

as closely as possible. Again, only general guidelines can be given. Each laboratory will have local circumstances which they alone can decide upon. Perform 20 analyses using the some control materials.

(i) Place the material randomly in different batches of serum. Avoid consistently using favoured positions such as close to standards.

(ii) Use standards and reagents as in routine conditions.

(iii) Use the personnel who will perform the routine analyses.

Calculate the mean value and standard deviation. Plot the results. Watch for 'outliers', this is unusually high or low results. Particularly watch for trends and if they occur, try to explain them.

Routine conditions variance will almost invariably be higher than the optimal conditions variance.

Stage 3, Routine conditions variance (unknown values) : Continue including control sera in each batch of analysis, but differ the dilution of the control serum so that the values are not known to the operator.

This can be done by appointing a person to prepare the control material for the operator and then passing it in a specimen tube, appropriately marked, to the operator. This techuique implies careful pipetting by the person appointed to make up the control material and careful documentation by both laboratory workers. The operator should not know the expected values.

Variation may be obtained by reconstituting the lyophilised control sera with known volumes between 9.0 ml and 11.0 ml in 0.5 ml stages in a random manner. Target values are available for each of the sera. Percentage deviation from the target values can be plotted against time. Such a plot will indicate drifts towoards higher or lower values.

$$\% \text{ Deviation from target value} = 100 - \frac{(T.V. - C.V.)\,100}{T.V.}$$

where, T. V. = target value, C. V. = calculated value = value found $\times \frac{10}{\text{reconstitution value}}$.

Watch for 'trends' in the graph and also 'outliers'. Search for the explanations of inconsistency.

Log book : During the assessmcnt of routine conditions variance (both known and unknown), a laboratory log book is essential. As experience with a particular method grows, what to record will become more obvious. Af first over recording is normal. It is not possible to lay down rigid rules as to what should be recorded, but the following arə guidelines :

(i) Changes of personnel

(ii) Changes in reagents

(iii) Changes in standards

(iv) Changes in instrumentation (eg breakdown and repair)

(v) Extremes in ambient temperature or humidity might be appropriate in some laboratories.

Control charts stating acceptable limits : Individual laboratories should establish their own criteria for acceptable limits for precision and accuracy.

Virtually all techniques need to be studied under the practical circumstances of a particular laboratory. When alterations of variance have been solved and the method performed under routine conditions without sudden or persistently gradual alterations in variance, then limits may be set up in each laboratory.

At the various stages of quality control, horizontal lines at ± 2 SD of the routine variance may be useful, but such statistics may alter from time to time and approximately one in twenty results is expected to fall outside these limits.

Calculating cumulative sum (CUSUM) and drawing cusum charts: Cumulative sum techniques are an aid to interpreting conventional control charts. They present the information in a manner which makes interpretation easier. An example of calculating cusum values is given in the Table. The method of plotting the results is shown in the Figures.

TABLE

CALCULATION OF CUMULATIVE SUMS

DAY	RESULT	RESULT – 100*	CUSUM
1	101	+1	+1
2	101	+1	+2
3	100	0	+2
4	98	– 2	0
5	103	+	+3
6	101	+1	+4
7	100	0	+4
8	102	+2	+6
9	98	– 2	+4
10	100	0	+4
11	101	+1	+5
12	99	– 1	+4
13	97	– 3	+1
14	100	0	+1
15	103	+3	+4
16	102	+2	+6
17	100	0	+6

TABLE *Cont.*

CALCULATION OF CUMULATIVE SUMS

DAY	RESULT	RESULT – 100*	CUSUM
18	99	– 1	+5
19	99	– 1	+4
20	99	– 1	+3
21	99	– 1	+2
22	101	+1	+3
23	101	+1	+4
24	100	0	+4
25	101	+1	+5
26	103	+3	+8
27	99	– 1	+7
28	100	0	+7
29	101	+1	+8
30	102	+2	+10
31	100	0	+10
32	98	– 2	+8
33	103	+3	+11
34	100	0	+11
35	100	0	+11
36	102	+2	+13
37	100	0	+13
38	98	– 2	+11
39	100	0	+11
40	102	+2	+13
41	98	– 2	+11
42	99	– 1	+10
43	100	0	+10

TABLE *Cont.*

CALCULATION OF CUMULATIVE SUMS

DAY	RESULT	RESULT - 100*	CUSUM
44	97	-3	+7
45	99	-1	+6
46	102	+2	+8
47	99	-1	+7
48	100	0	+7
49	102	+2	+9
50	100	0	+9

*The value of 100 is the arbitrarily chosen cusum reference value.

Consider the daily results of the analysis of a control serum as shown in the Table. In the first column the analytical results obtained are recorded. In order to prepare a cusum chart a value in the middle of the range of results is chosen. For reasons discussed later, the closer this value is to the best estimated (or mean value) the better, but at first, when the mean is not known any arbitrary value can be chosen. In the illustrations a value of 100 was chosen. This value is substracted from the observed value and the result recorded in column 3. In the 4th column the numbers are cumulatively summed starting at day 1, adding day 2 and so on upto day 50. The graphical plot of this cumulatively sum against the sequential number of the analysis is a cusum graph.

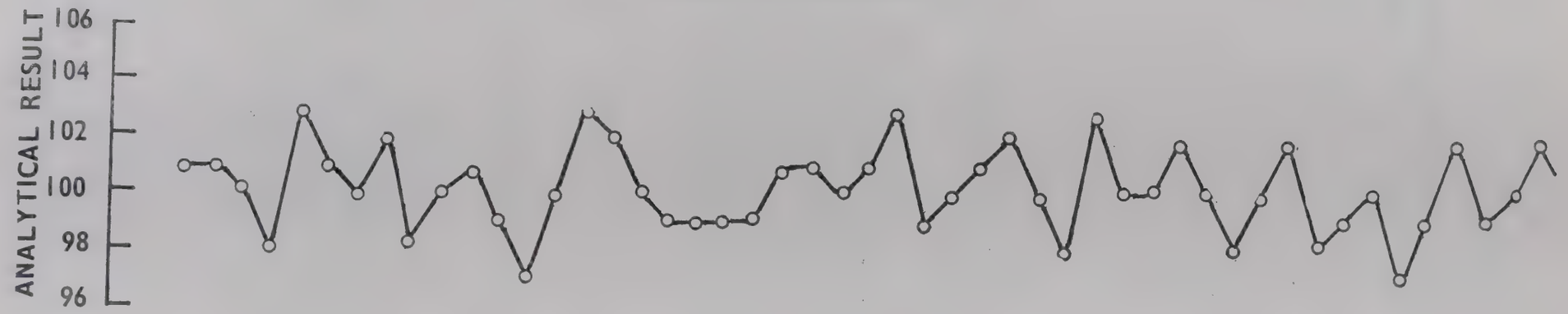
Using such a graph, drifts in the results can be detected more easily by eye than using a conventional control chart.

In cusum graphs general trends in the slope of the graph and changes in that slope indicate changes in the results with respect to the chosen reference value.

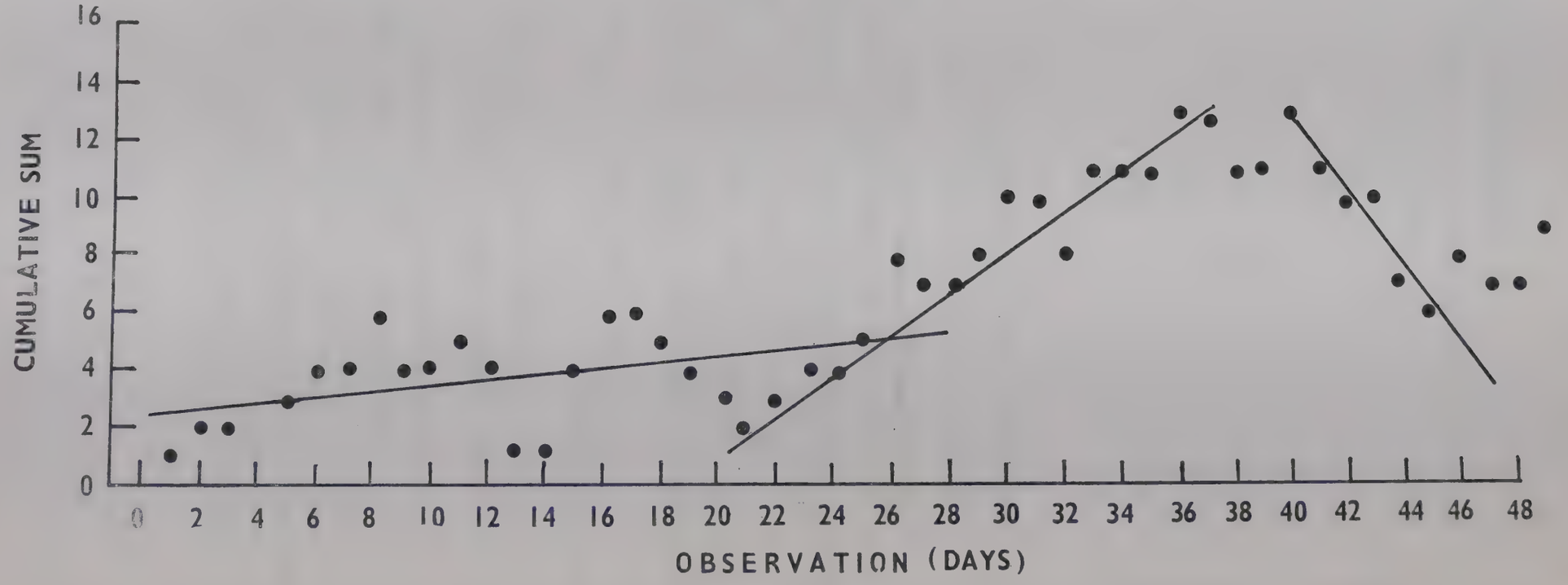
In the graph the slope over the first 25 days is horizontal. There is a change from approximately the 25th to the 35th day, the method giving, higher results. Such a trend is not obvious by inspection of the conventional control chart. From the 40th to 45th day the method gives lower results. In the cusum plot the trends have been illustrated by free hand drawn first lines. The slope of such trends is dependent on the chosen reference value. The changes in slopes are not dependent on the choice. A convenient scale to use in plotting cusum charts is that the horizontal scale of one day should equal a vertical scale of approximateiy 2 SD for the method.

CONVENTIONAL CONTROL CHART

(Chosen ref. value 100)



CUSUM GRAPH



SECTION - B

METHODS

METHODS

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I. FOOD ANALYSIS

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1. PREPARATION OF SAMPLE

Pulses and cereals : The sample is cleaned free of sand, stone and other extraneous material and powdered. Use of iron grinder should be avoided when estimation of iron content is needed.

Fruits and vegetables : Fruits and vegetables which are small in size can be taken as such to prepare samples for analysis. But those which are big should be cut and portions ($\frac{1}{2}$ to $\frac{1}{4}$) of the edible material is used. The nonedible portions are rejected and the edible portions cut into small pieces. Samples for the analysis are taken from a mixture of these pieces by quartering. It is preferable to use stainless steel trays, knives and scissors for the preparation of the sample.

Flesh foods : Either the whole tissue or a representative portion of it is minced with scissors and then blended with double its weight of water. Portions of the slurry are then removed into weighed containers which are then reweighed to determine the amount of tissue used

Liquid foods : Samples are mixed thoroughly and filtered through a muslin cloth. Portions of this mixed sample are then taken for analysis.

Aliquots are taken from the representative samples for analysis.

2. MOISTURE

About 10 g of the material are weighed into a weighed moisture box and dried in an oven at 100 to 105°C and cooled in a desiccator. The process of heating and cooling is repeated till a constant weight is achieved.

$$\text{Moisture } \% = \frac{\text{Initial weight} - \text{Final weight}}{\text{Weight of the sample}} \times 100$$

3. PROTEIN

The protein content of a foodstuff is obtained by estimating the nitrogen content of the material and multiplying the nitrogen value by 6.25 (a factor of 5.7 used for wheat). This is referred to as crude protein content since the nonprotein nitrogen (NPN) present in the material is not taken into consideration. True protein nitrogen can be determined by subtracting NPN from the total nitrogen. The estimation of nitrogen is done by Kjeldahl method (Refer Proteins amino acids and metabolites).

4. FAT

Fat is estimated as crude ether extract of the dry material.

The dry sample (5-10 g) is weighed accurately into a thimble and plugged with cotton. The thimble is then placed in a Soxhlet apparatus and extracted with anhydrous ether for about 16 hr. The ether extract is filtered into a weighed conical flask. The flask containing the ether extract is washed 4 to 5 times with samll quantities of ether and the washings are also transferred. The ether is then removed by evaporation and the flask with the residue dried in an oven at 80 - 100°C, cooled in a desiccator and weighed.

$$\text{Fat content (g/100 g sample)} = \frac{\text{Wt of ether extract} \times 100}{\text{Wt of the sample (equivalent to fresh sample taken)}}$$

5. CRUDE FIBRE

About 2 – 5 g of moisture and fat free sample are weighed into a 500 ml beaker and 200 ml of boiling 0.255 N (1.25%W/V) sulphuric acid added. The mixture is boiled for 30 min keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker helps smooth boiling). At the end of this period, the mixture is filtered through a muslin cloth and the residue washed with hot water till free from acid. The material is then transferred to the same beaker and 200 ml of boiling 0.313 N (1.25%) NaOH added. After boiling for 30 min (keeping the volume constant as before) the mixture is filtered through muslin cloth. The residue is washed with hot water till free from alkali, followed by washing with some alcohol and ether. It is then transferred to a crucible, dried overnight at 80-100°C and weighed (We). The crucible is heated in a muffle furnace at 600°C for 2 to 3 hr, cooled and weighed again (Wa). The difference in the weights (We-Wa) represents the weight of crude fibre.

$$\text{Crude fibre (g/100 g sample)} = \frac{100 - (\text{moisture}^* + \text{fat}^*) \times \text{Wt of fibre}}{\text{Wt of sample taken (moisture and fat free)}}$$

* g/100 g sample.

6. ASH

About 5-10 g of the sample are weighed accurately into a tared platinum (or porcelain) crucible (which has previously been heated to about 600°C and cooled). The crucible is placed on a clay pipe triangle and heated first over a low flame till all the material is completely charred, followed by heating in a muffle furnace for about 3-5 hr at about 600°C. It is then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible is again heated in the muffle furnace for $\frac{1}{2}$ hr, cooled and weighed. This is repeated till two consecutive weights are the same and the ash is almost white or greyish white in colour.

$$\text{Ash content (g/100 g sample)} = \frac{\text{Wt of the ash}}{\text{Wt of the sample taken}} \times 100$$

7. CARBOHYDRATES

The content of the available carbohydrate is determined by difference, ie., by subtracting from 100 the sum of the values (per 100 g) for moisture, protein, fat, ash and crude fibre.

8. MINERALS

Calcium, magnesium, copper, iron, phosphorous, chloride, sulphur, fluorine and iodine (Refer Minerals).

9. VITAMINS

Thiamine, riboflavin, niacin, ascorbic acid and carotenoids (Refer Vitamins coenzymes and metbolites).

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1. TOTAL PROTEINS

a. Macro-Kjeldahl method

Principle :

The estimation of nitrogen is done by Kjeldahl method which depends upon the fact that organic nitrogen when digested with sulphuric acid in the presence of a catalyst (selenium oxide, mercury or copper sulphate) is converted into ammonium sulphate. Ammonia liberated by making the solution alkaline is distilled into a known volume of a standard acid, which is then back-titrated. The protein content is obtained by multiplying the nitrogen value with 6.25.

Reagents :

1. Digestion mixture : 98 parts K_2SO_4 + 2 parts $CuSO_4$
2. 40% NaOH
3. N/10 NaOH
4. N/10 H_2SO_4
5. Methyl red indicator : 0.1 g of the indicator dissolved in 60 ml of alcohol and water added to make to 100 ml.

Procedure :

The sample (0.5-2.0 g) is weighed into a dry Kjeldahl flask. About 5 g of digestion mixture and 20 ml of pure conc. H_2SO_4 are added to the same sample and the mixture digested by heating for 4 to 5 hr. Glass beads are added to prevent bumping. After the contents of the flask become clear, the digestion is continued for atleast 1 hr. The contents of the Kjeldahl flask are cooled, diluted with distilled water and the mixture made alkaline by adding excess of 40% NaOH (about 75 ml). A small quantity of pumice powder is added to prevent bumping during distillation. The ammonia liberated is distilled into a receiver containing 25 ml of N/10 H_2SO_4. The excess of acid in the receiver is back-titrated against N/10 NaOH using 3 drops of methyl red indicator.

A reagent blank is similarly digested and distilled. This titre value is subtracted from the value obtained for the sample to get the true titre value 'b'.

Calculation :

If 'a' g of the sample are taken and if 'b' and 'c' ml of alkali of normality 'd' are required for back-titration and to neutralise 25 ml of N/10 H_2SO_4 respectively, then the protein content in g/100 g sample is $\frac{(c-b) \times 14\ d \times 6.25}{a \times 1000} \times 100$

Note :

True nitrogen can be determined by subtracting NPN (which is estimated by precipitating the protein in the sample with trichloracetic acid, tungstic acid or copper hydroxide and determining the residual nitrogen in the protein free filtrate) from the total nitrogen.

b. Micro-Kjeldahl method

Principle :

The nitrogenous compounds of plasma or serum are converted into ammonium sulphate by boiling with concentrated sulphuric acid. It is subsequently decomposed by addition of excess of alkali and the liberated ammonia absorbed into a boric acid solution containing bromocresol green indicator by steam distillation. Ammonia forms a loose compound, ammonium borate with boric acid which is titrated directly against standard sulphuric acid.

Reagents :

1 40% NaOH in distilled water.

2. 2% Boric acid : Dissolve 20 g of reagent grade boric acid in about 500 ml of hot distilled water and add 2 ml of 0.1% bromocresol green in alcohol (or aqueous solution of the sodium salt) and finally make up the volume to 1 l with distiiled water.

3. 0.01 N H_2SO_4 (standardised).

Procedure :

Transfer plasma (0.2 ml) or any other biological material into a 50 ml long necked micro-Kjeldahl flask and wash the sides of the flask with distilled water. Add 2 ml of concentrated sulphuric acid and digest on a heated sand bath or on the microdigestion unit. When charring begins and white fumes appear in the flask stop digestion for a while to allow the flask to cool and add a few drops of hydrogen peroxide. Continue the digestion till the solution becomes clear. If necessary repeat the addition of hydrogen peroxide and digestion, to hasten the process. Add distilled water and boil for a few minutes and allow the flask to cool.

Take 10 ml of the boric acid solution in a 100 ml conical flask and place in such a way that the tip of the condenser outlet of the steam distillation apparatus dips below the surface of the boric acid solution. If the colour of the boric acid solution has faded, 2 or 3 drops of bromocresol green indicator may be added. Transfer the digested sample completely by means of repeated rinsings to the chamber of the steam distillation apparatus. The chamber should be previously cleared off any contaminating ammonia by repeated washings. Add about 8 ml 40% NaOH (or a quantity enough to make the mixture alkaline) to the digest in the chamber. Start the steam generation and make the distillation set airtight. Steam distill till about 30 ml distillate is collected into receiving flask. Lower the receiving flask and stop the steam generation. Wash the condenser outlet tube into the receiving flask with a little distilled water.

The solution in the receiving flask is coloured blue at this stage. Titrate the contents against 0.01 N H_2SO_4 till the original green colour is obtained.

Run the blank preparation through all the steps by taking distilled water in place of sample.

Calculations :

1 ml of 0.01 N H_2SO_4 = 0.00014 g nitrogen. Since average nitrogen content of most proteins is 16%, 1 g nitrogen = $\frac{100}{16}$ g protein.

If the titre value for sample minus the titre value of blank is X, protein content of the sample in 100 ml $= X \times 0.00014 \times \frac{100}{16} \times \frac{100}{0.2} = X \times 0.4375$ g.

Reference :

Practical Physiological Chemistry, Hawk and Oser (Eds.) 14th Edn. p. 1214 (1965).

c. Biuret method

Principle :

The - CONH groups in the protein molecule react with copper sulphate in alkaline medium to give purple colour which is then read at 540 nm.

Reagents :

1. Biuret reagent : Dissolve 4.25 g of potassium sodium tartarate ($KNaC_4H_4O_6.4H_2O$), 1.5 g of cupric sulphate ($CuSO_4 . 5H_2O$) and 2.5 g potassium iodide in about 500 ml of distilled water. Dissolve 4 g of NaOH in the solution and make up the volume to 1 l.

2. Standard : The standard protein solution may be either a pooled normal human serum (standardised by Kjeldahl method) or a solution of pure albumin in saline.

Procedure :

To 0.1 ml aliquots of standard, test plasma and blank (saline or distilled water) add 5 ml of biuret reagent. Mix well and keep for 30 min. Read absorbances of test and standard against blank at 540 nm.

Calculation :

$$\text{Total protein g/100 ml} = \frac{\text{reading of test}}{\text{reading of standard}} \times \text{concentration of standard.}$$

Reference :

Am. J. Clin. Path. **16**, 40 (1946).

d. Lowry's procedure

Principle :

The final colour is a result of, (a) biuret reaction of protein with copper ion in alkali and (b) reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

Reagents :

1. 4% Na_2CO_3
2. 0.5% $CuSO_4.5H_2O$ in 1% potassium sodium tartarate
3. Alkaline copper solution. Mix 50 ml of reagent 1 with 2 ml of reagent 2 (prepare fresh every day).
4. 0.1/N NaOH

5. Diluted Folins' reagent : Dilute Folin-Ciocalteau reagent (purchased from V. P. Chest Institute, Delhi) with an equal volume of 0.1 N NaOH
6. Standard protein solution : The standard solution is prepared with bovine serum albumin to have a concentration of 100 μg protein/ml.

Procedure :

The test sample contcining about 50-100 μ g protein/ml is taken at three levels, 0.5, 1.0 and 1.5 ml. The necessary amount of 0.1 N NaOH is added to bring the volume to 1.5 ml. To this 1.5 ml. of reagent 3 is added. Each tube is shaken and allowed to stand for 10 min after which exactly 0.15 ml. of diluted Folin's reagent added with continuous shaking. The tubes are allowed to stand for half an hr and then read at a wavelength of 750 nm. For more concentrated solutions, the readings may be kept in a workable range by reading it at a wavelength of 500 nm.

The unknown sample is then read off the standard curve and the necessary calculations made.

Reference :

1. Methods in Enzymology, Vol. III, p 448 (1957)
2. J. Biol. Chem. **193**, 265 (1951).

e. Modified Lowry's procedure

Principle :

Same as for Lowry's procedure.

Reagents :

1. Alkaline copper reagent : Add 10 ml 1.0% potassium tartarate and 10 ml 0.5% $CuSO_4$ with mixing to 20 ml of 10% NaOH. Then add 50 ml of 20% sodium carbonate, mix and make upto 100 ml with water.
2. Phenol reagent : Dilute 1 volvme of Folin-Ciocalteau reagent (2.0 N), with 16 volumes of distilled water just before use.
3. Protein standard : Dilute bovine serum albumin (BSA; fraction V, Sigma), stock solution with 1% sodium dodecyl sulfate (SDS) to give a concentration of 100 μg/ml. Use 10-50 μg for the standard curve.

Procedure :

To 0.5 ml of sample, blank or standard taken in duplicate add 0.5 ml of alkaline copper reagent, mix and let stand undisturbed for 10 min. Then add 2.0 ml phenol reagent forcibly and rapidly to each tube. Mix immediately and heat in a water bath at 55°C for 5 min. Cool in running water and read the absorbance of samples and standard at 650 nm against blank. Calculate the protein content of samples by comparing with standard.

Note :

Very dilute suspensions of organelle preparations for protein estimation by Lowry's method may be prepared by taking a small aliquot (Ca 50 - 100 μl) and making it up with 1.0%SDS to 5 ml.

Reference :

Anal. Biochem. 51, 654 (1973).

f. Electrophoretic separation

Principle :

Proteins carry charge at pH other than their isoelectric pH. Above the isoelectric pH they are negatively charged and below that they are positively charged. Hence, in an electrical field theymove differently depending on the net charge they carry in a given buffer system.

Materials :

1. Buffer solution, Veronal, pH 8.6: 2.76 g diethyl barbituric acid, 15.40 g sodium diethyl barbiturate, dissolved in 1.0 l.
2. Samples : 6–8 μl serum.
3. Paper : Whatman No. 1 strips, 3.0 cm wide by 30.6 cm long. 8 strips required.
4. Wicks.
5. Current : 2.5 ma held constant, per cell. (5.0 ma if two cells are connected to a single Duostat power supply).

Procedure :

The centre of the paper strip is marked and the strip is moistened with the buffer. The strip is then kept in the electrophoretic apparatus and the serum is applied at the marked portion of the strip with lambda pipette. The ends of the strip are connected by paper wicks to the buffer solution in the tanks. The run is started by applying 2.5 ma direct current. The run is terminated after 16-18 hr in a horizontal apparatus and 4-5 hr in a vertically run apparatus. Dry the strip at 100-130°C for 10 min and proceed with staining as follows :

Staining :

1. Pre-rinse : Methanol, purified grade 1 l. Immerse strips for 6 min in order to prevent carrying buffer salts into the dye solution. This pre-rinse may be reused several times.
2. Stain : 0.1 per cent by weight, bromphenol blue in methanol. Immerse strips for 30 min without agitation. The stain solution may be reused three or four times.

Destaining :

5% glacial acetic acid (V/V). Immerse strips for 6 min without agitation. Repeat this step for 3 times with fresh 5% acetic acid.

Drying :

Dry the strips at 120-130°C for 15 min.

Scanning :

The dried strips are exposed to ammonia vapour for 15 min before scanning.

Reference :

Beckman Electrophoresis manual.

g. Sephadex gel filtration :

Separation of the basic protein from the albumin fraction of milk is given here as an example of the use of sephadex in the separation of proteins.

Procedure :

Ten g of sephadex G-150 is allowed to swell in a large excess of water for one day with occasional shaking. Then it is packed into a column (1.5 x 50 cm) having a glass wool plug at the bottom. While packing the column care is taken to prevent the formation of air bubbles in the column. Then the column is washed with water for 1 to 2 hr. When the water on the column is completely drained off, a circular filter paper is kept on top of the packed gel column to protect the surface. Three ml of human milk albumin fraction having the basic protein is applied on the column erected on an automatic fraction collector. When the miniscus of the solution touches the surface of the solid sephadex column, 3 ml of water is added and allowed to drain as mentioned above. This is followed by another 5 ml of water. The fraction collector is adjusted to collect 60 drops in each tube.

The optical density of the collected fractions is taken in a Beckman spectrophotometer at 280 nm. A graph is plotted with optical density on the Y-axis and tube number on the X-axis. Usually, the milk albumin separate into 3 fractions and three peaks will be observed. The fractions belonging to each peak is collected separately and freeze dried. The third peak contains basic protein with small amounts of albumin. The separation of free basic proteins is achieved by loading sephadex G-75 column with the protein obtained from the third peak. The elution is then carried out with water as detailed before. Eluent corresponding to second peak contains the basic protein in pure form.

References :

1. Nature, **183**, 1657 (1959).
2. J. Chromat. **3**, 330. (1960),
3. Anal. Chem. **35**, 1950. (1963).

2. IMMUNOGLOBULINS

Principle :

The size of the precipitation ring formed due to antigen - antibody reaction is used in determining quantitatively the immunoglobulin levels in tissue fluids.

Reagents :

1. Monospecific goat antiserum to human IgG, IgA and IgM (obtained from Hyland Laboratories or NBC).
2. Standard human serum of known immunoglobulin concentrations (obtained from WHO international immunological reference centre).
3. Noble agar - 1.5% and 1.0%
4. 1% acetic acid in methanol

Procedure :

Preparation of agar plates : Glass plates are cleaned free of dust and grease. Precoating of plate is done by using 1.0 % agar in distilled water (boiled for dissolving and cooled to just above gelling point

46 - 48°C). The plates are dried completely. The precoating minimises movement of agar gel later poured onto the slide and changes of reactant (antiserum or serum) leakage between the gel and the slide.

Agar (1.5%) is prepared in 12 ml of distilled water and heated to dissolve completely. Then the agar solution is cooled to gelling point and antisera to be added is determined by trials with different levels of antisera and formation of precipitation rings. Too much of antisera can lead to dissolution of antigen and too little will not give clear cut rings.

After adding and mixing the antisera the agar gel is poured on to the plate to form a layer 2 mm thick. Surface tension keeps the agar from running off the slide. After the agar gel sets in, wells are punched in at regular intervals using negative suction with the pasteur-pipette.

Sampling and quantitation : Standard serum and test sera are diluted appropriately depending on the known concentration of immunoglobulin in serum. For IgG, dilution is to the extent of 1:10 or 1:15, for IgA, 1:4 and for IgM, sera are used straight. The dilution is done with 2% BSA and a drop of 1% sodium azide is added to prevent fungal growth. After use, the sera can be preserved at 4°C for two weeks.

The standard serum is placed in the first and the last four wells at different concentrations ranging from 2-8 µl for IgG and IgA or 4-12 µl in case of IgM. The test samples are also placed in the wells at three levels. In the plates which are used, 13 samples at 3 different levels and two sets of standard can be assayed.

After sampling the plates are left in a humid chamber (a petridish containing wet filter paper pieces) for 48 hr at room temperature.

After 48 hr, plates are removed, soaked in distilled water for 2 hr, to get rid of soluble proteins and excess antiserum. The antigen-antibody reaction is visible with well defined margins. If the rings are not very clear, soaking the plates in 1% acetic acid in methanol for a few min demarcates the ring margins clearly.

Both the vertical and horizontal diameters of rings are measured on a microscope using the vernier scale attached to the mechanical stage. Dark field illumination is used (blocking the con densor with black paper and illuminating the slide from the top at 45° to the objective). The average of these two diameters is calculated. A standard curve is drawn using the different levels of standard serum against the ring diameter. The concentration of immunoglobulin in test sera is read from the standard graph and corrected for the dilution. The values of immunoglobulins are expressed as percentage of standard serum, as the reference serum has not yet been assayed quantitatively for different immunoglobulins.

Note:

Serum transferrin and ceruloplasmin levels can be determined in a similar way using antisera for human transferrin and ceruloplasmin. The procedure is similar to that described for the immunoglobulins.

Reference :

J. Lab. Clin. Med. **52**, 784 (1958).

3. ALBUMIN

a. Micro - Kjeldahl method

Reagents :

As given for total proteins and 28% sodium sulphite.

Procedure :

Add 4.8 ml of 28% sodium sulphite solution to 0.2 ml of plasma. Mix by rotating the tube for 2 to 3 min. Filter the solution through a Whatman No. 40 filter paper. Take 2.5 ml of the filtrate and proceed the digestion, distillation and titration as given under total proteins.

Calculation :

Albumin content of plasma = X x 0.875g /100 ml.

b. Dye binding method

Principle :

The increase in absorbance of BCG at 630 nm on binding to serum albumin at pH 4.2 is directly proportional to the concentration of albumin.

Reagents :

1. Buffered BCG dye solution : Dissolve 8.85 g of succinic acid, 108 mg of BCG (sodium salt) and 100 mg of sodium azide in about 950 ml of water. Add 4 ml of 30% Brij-35, adjust the pH to 4.2 and make up to 1 l with water (Stable for atleast 5 months at room temperature).

2. Standard : A pooled serum sample may be used as standard after estimation of albumin by Kjeldahl method (Bovine serum albumin is unsatisfactory but human serum albumin fractions of highest purity available commercially may be used).

Procedure :

Take 3.0 ml buffered dye solution in a series of tubes. Add 10 μl of serum sample vortex and read immediately (within 30 sec) the absorbance at 630 nm against blank dye solution. Analyse in duplicate and calculate the albumin content by comparison with standard run similarly.

Note :

Although the zero time absorbance method described above is a true estimate of albumin, significant errors may result if readings are taken after 60 sec. Readings must therefore be taken immediately.

References :

1. Standard Methods of Clinical Chemistry, G. R. Cooper (Ed). Academic Press, New York, Vol. 7, p. 175 (1972).
2. Clin. Chem. **22**: 616 (1976).

4. HAEMOGLOBIN

a. Cyanmethaemoglobin method

Principle :

Haemoglobin is converted into cyanmethaemoglobin by the addition of KCN and ferricyanide. The

colour of cyanmethaemoglobin is read in a photoelectric colorimeter at 540 nm against a standard solution. Since cyanide has the maximum affinity for Hb this method estimates the total Hb.

Reagent:

Drabkin's solution : Dissolve 0.05 g of KCN, 0.20 g of potassium ferricyanide and 1.00 g of sodium bicarbonate in 1 l of distilled water.

Procedure :

Blood 20 μl is transferred with the help of a haemoglobin pipette into a test tube containing 5 ml of Drabkin's solution. The tubes are mixed and readings taken in a photoelectric colorimeter (Klett-Summerson) using 54 filter. The reagent blank (Drabkin's diluent) is adjusted to zero.

Construction of a standard curve :

(i) Since the amount of iron present in Hb per unit weight is constant, one can calculate the Hb concentration of a given blood sample by determining its iron content. This blood sample can be used as a reference standard for Hb estimation.

(ii) A standard curve can also be constructed by using the standard cyanmethaemoglobin solutions (supplied by BDH or V. P. Chest Institute, Delhi).

Standard solution (ml)	Drabkin's diluent (ml)	Hb conc. (%)
5	0	100
5	2.5	67
5	5.0	50
5	7.5	40
5	10.0	33

The suppliers of the standard solutions mention the concentration of the standard on the ampoule. The corresponding blood Hb in g/100 ml can be obtained by multiplying the concentration on the ampoule by the dilution factor (251).

Note :

1. Drabkin's solution should be stored in amber coloured bottle. If any precipitate is formed the reagent should be discarded.
2. Since the dilution is enormous (251 times) accurate measurement of 20 μl of blood is absolutely essential for reproducibility. Hb pipettes must be checked for their accuracy by weighing pure mercury upto the mark.

Reference :

Practical Haematology, J. V. Dacie and S. M. Lewis (Eds), Churchill Livingston, V Edn p32 (1975)

b. Filter paper technique

The cyanmethaemoglobin method has been modified to suit the needs of field investigations. Twenty μl of blood are measured accurately from a Hb pipette and delivered on to a Whatman No. 1 filter paper disc. The filter paper is air-dried, labelled and brought to the laboratory.

In the laboratory the portion of the filter paper containing the blood is cut out and dipped in 5 ml of Drabkin's solution taken in a test tube. Wait for 30 min mix the contents on a vortex mixture and take the readings with 54 filter as described above. The filter paper disc containing blood can be stored upto one week.

Reference :

Annual Report, National Institute of Nutrition, p. 153 (1974).

c. Wong's method

(Refer Minerals under Iron)

5. CERULOPLASMIN

Principle :

Serum ceruloplasmin has oxidase activity. Hence, ceruloplasmin is estimated in terms of its para-phenylenediamine oxidase activity at an optimal pH and temperature.

Reagents :

1. 0. 1 % para-phenylenediamine (PPD) in acetate buffer at 37°C.
2. Acetate buffer : Twenty ml of glacial acetic acid and 163 g sodium acetate (CH_3 COONa, $3H_2O$) are dissolved in 1 l glass distilled water, ionic strength 1. 2, pH 5. 2.
3. 0. 02 % sodium azide solution.

Procedure :

To 1 ml of freshly prepared 0. 1 % solution of PPD in acetate buffer at 37°C, 0.1 ml of serum or plasma is added and incubated at 37°C for 15 min. To this about 5 ml of 0. 02 % sodium azide solution is added to stop the reaction. The OD is measured in Beckman DU, spectrophotometer at 525 nm.

Serum blank : Take reagent blank containing azide. Add 0. 1 ml serum and incubate for 15 min.

Reagent blank : One ml of reagent without serum is incubated for 15 min at 37°C. Then azide is added.

Calculation :

The ceruloplasmin in the sample is expressed in terms of OD units of PPD-oxidase activity.

Note :

The OD is to be determined within 30 min if a water blank is used. If a reagent blank is prepared and colour is read against it, the OD can be determined within 5 hr (to prevent errors due to auto-oxidation of PPD). A serum blank may not be necessary if a reagent blank is used.

Reference :

Clin. Chem. 4, 519 (1958).

6. ALPHA-FETOPROTEIN

Principle :

The estimation of alpha-fetoprotein is based on the principle of radioimmunoassay.

Reagents :

1. 0.07 M barbital buffer, pH 8.6 : Dissolve 2. 5g of acid barbital and 14.4 g of sodium barbital in 1 l of glass distilled water. Add 100 mg of merthiolate as preservative.

2. 0.5 M phosphate buffer, pH 7.5 : Mix 16.0 ml of 1. 0 M NaH_2PO_4. $2H_2O$ (15.6 g in 100 ml of water) and 81.0 ml of 1.0 M Na_2HPO_4 (71 g in 500 ml of water) and dilute to 200 ml with water.

3. 0.05 M phosphate buffer, pH 7.5 : Dilute the above buffer 1 in 10. All reagents except for radioactive iodine should be dissolved in this buffer.

4. Chloramine T : Dissolve 50 mg chloramine T in 10 ml 0.05 M phosphate buffer, pH 7.5, just before use.

5. Sodium metabisulphite : Dissolve 24 mg sodium metabisulphite in 10 ml 0.05 M phosphate buffer, pH 7. 5, just before use.

6. Alpha-fetoprotein soultion : Exactly 10 μg of alpha-fetoprotein in 10 μl of glass distilled water. (This was supplied by International Agency for Research on Cancer).

7. Potassium iodide : Dissolve 100 mg of potassium iodide in 10 ml of 0.05 M phosphate buffer, pH 7.5. This solution is stable for several days at room temperature.

8 Radioactive iodine : Carrier free ^{125}I as sodium iodide is supplied from Radiochemical Centre. Amersham, England. The specific activity 13-17 mCi/μg.

9. 2% albumin solution : Dissolve 2g of BSA (fraction V) in 100 ml 0.07 M barbital buffer.

Procedure :

Sephadex column : G-50, G-200 (Refer RIA of Hormones).

Protocol for iodination : Add the following reagents in order of the sequence given below and shake well after each addition.

10 μl of alpha-fetoprotein, 20 μl of ^{125} I, 10 μl of 0.5 M phosphate buffer, pH 7. 5, 10 μl of chloramine T.

Allow the reaction for 30 sec, then add, 100 μl of sodium metabisulphite, 200 μl of potassium iodide in two portions and 100 μl of normal human plasma.

Transfer the reaction mixture on to the sephadex G-50 column by means of a capillary pipette. Add few drops of potassium iodide solution to the reaction vial and transfer on to the column. The empty

reaction vial and pipette should then be counted. Elute the column with 0.05 M phosphate buffer containing normal saline solution and collect 1 ml fractions at the rate of 1 ml/1.5 min. Collect 20 such fractions and count the radioactivity. The protein peak appears at 4–6 ml and the salt peak of iodide at 15-20 ml.

Purification of ^{125}I alpha-fetoprotein : This is done on Sephadex G-200 column (Refer RIA of Hormones).

Protocol for incubation : To 0.1 ml of standard alpha-fetoprotein or serum after appropriate dilution add 0.1 ml of anti-alpha-fetoprotein (1 : 1000), 0.5 ml of 0.05 M phosphate buffer. Mix and incubate at 4°C for 18 hr. Add 0.1 ml of ^{125}I alpha-fetoprotein (10, 000-15, 000 cpm). Incubate for 18 hr at 4°C. Finally, add 0.1 ml of guinea pig anti-rabbit γ-globulin (1 : 2 or 1 : 3) and 0.1 ml of normal rabbit serum. Mix and incubate at 4°C overnight.

Centrifuge the tubes at 3000 rpm for 20 min at 4°C. Decant the supernatant fluid from each tube carefully without disturbing the precipitate into another clean rimmed test tube. Count the precipitate (bound ^{125}I alpha-fetoprotein) and the supernatant (free ^{125}I alpha-fetoprotein) separately in a well type scintillation γ-counter. Calculate the bound/free ratio (for the method of calculation refer Hormones).

Reference :

1. Br. J. Obstet. Gynecol. **82**, 669 (1975).
2. Nature, **194**, 495 (1962).
3. Am. J. Obstet. Gynecol. **127**, 394 (1977).

7. SERUM RETINOL BINDING PROTEIN

Principle :

Refer Section A under 'Saturation Analysis'.

Reagents :

1. Barbital buffer : Mix diethyl barbiturate 9 g, hydrochloric acid 0.1 N 65 ml and sodium azide 0.5 g. Volume is made upto 1 l with distilled water and pH to 8.6 with HCl.
2. Serum of known RBP conc : 6 mg/100 ml of serum
3. Specific anti-RBP serum : This antibody is diluted 1 : 10 with barbital buffer and stored at 4°C.
4. 1 % Sodium azide
5. 4 % Tannic acid

Procedure :

Preparation of agar plate : About 159 mg agar powder is weighed into a 15 ml test tube. Ten ml of barbital buffer and 1 ml of sodium azide are added. After mixing, the tube is kept in boiling water bath till it forms a clear solution. The solution is then brought down to 60°C.

Preparation of agar-antiserum mixture: To 0.12 ml of diluted antibody (1:10 dilution) enough barbital buffer is added to make the volume to 2.0 ml. This solution is brought to 60°C by keeping in hot

water bath just before mixing with agar solution. The agar solution is mixed with antibody and agar-antibody mixture is then poured on to a clean glass plate (8 X 8 cm) kept on horizontal level. The plate is left at room temperature for 10 min and then transferred to 4°C for 30 min.

Application of antigen : Five rows of 5 wells each are punched out uniformly in the gel with the help of water suction using metallic cannula. The volume of punched well is approximately 10 μl.

Undiluted standard serum of known RBP concentration (antigen) is applied by using 10 μl pipette in the first row in order of 2, 10, 4, 8 and 6 μl from left to right. The final volume is made up to 10 μl by adding distilled water in all the wells. Five to 10 μl of unknown serum is spotted in rest of the wells in duplicate and final volume made upto 10 μl by adding water.

Incubation : Before keeping the plate for incubation, the left upper corner of the agar is cut in oblique fashion to identify the starting point. Agar plate is then kept for incubation in a petridish containing moist tissue paper for 48 hr.

Staining of plate : After incubation, the plate is immersed in barbital buffer for 20-30 min. Two such washings are done to remove excess of antibody. Following this, 4% tannic acid is poured over the plate and left for 20-30 min. Excess of tannic acid is removed by again washing the plate 2-3 times with barbital buffer. Readings of the diffusion rings (diameter) are taken with the help of caliberated scale.

Standard graph is constructed by plotting diameter readings of the rings against the concentrations of standard antigen used on a log graph. Diameter readings of sample rings are read against standard curve and the concentrations of RBP calculated. The RBP levels are expressed as μg/ml.

Reference :

Imm. Chem. **2**, 235 (1965).

8. CELLULAR RETINOL BINDING PROTEIN

Principle :

The cellular retinol binding protein (CRBP) is assayed by incubating the cytosol fractions of tissues with ^{3}H-retinol and by separating the bound retinol. The nonspecific binding is measured by running parallel incubations with excess cold retinol and the difference between total and nonspecific binding represents the true concentrations of CRBP.

Reagents :

1. 0.05 M Tris-HCl buffer, pH 7.4 : Take 6.057 g Tris and 100 mg of thiomersal and dissolve in about 800 ml of glass distilled water. Adjust the pH to 7.4 with HCl and make up volume to 1 l.

2. Dilute ^{3}H-retinol in dimethylsulfoxide (Sp. act. 1.25 Ci/mmole to give 40 pmoles in 5μl (about 26,000 cpm).

3. Retinol: Eight nmoles/5μl in dimethylsulfoxide.

4. Cytosol preparation : The organs are collected from adult rats and stored at -40°C untill use. The organs are homogenised in 0.05 M Tris-HCl, pH 7.4 (2-4 ml/g tissue) with a glass-teflon tissue homogeniser. The homogenates are centrifuged at 31,000 g for 10 min in refrigerated centrifuge. The

supernatant is collected and centrifuged again at 105,000 g for 60 min at 4°C in an ultracentrifuge. The supernatant (cytosol) is used for CRBP estimation after exposing to UV light in cold to destroy the endogenous retinol.

Procedure :

Aliquots (1 ml) of cytosol are mixed with 5 μl of ^{3}H-retinol, incubated overnight in dark at 0-4°C. The bound retinol in these tubes represents total binding capacity. Parallel incubations are run with the addition of 200 fold excess (8 nmoles in 5 μl of dimethylsulfoxide) of cold retinol to get nonspecific binding. Aliquots (0.1 ml) of the incubation mixture are applied to columns of sephadex G-25 (1.1 x 5.5 cm) equilibrated with 50 mM Tris-HCl (pH 7.4). The samples are eluted with the same buffer (flow rate about 25 ml/hr) and 0.5 ml fractions are collected. The fractions are transferred to counting vials, mixed with 5 ml of Bray's mixture and counted for radioactivity in a liquid scintillation counter. The bound counts are eluted in the void volume and free ^{3}H-retinol is separated in later fraction. The difference between the total and nonspecific binding represents specific binding and is a measure of true CRBP level.

Reference :

J. Biol. Chem. **253**, 828 (1978).

9. COLLAGEN

Principle :

Collagen forms the major portion of the dermal protein. Even though mature collagen is insoluble, there are fractions of collagen which can be extracted into neutral salt solution and mild acid solution.

Sodium chloride concentration ranging from 0.15-1.0 M is generally used for extracting the salt soluble collagen. Collagen soluble in 0.15 M NaCl represents newly synthesised collagen. After preparing the salt soluble collagen, the residue is extracted with 0.2 M sodium citrate, pH 3.5 or 0.5 M acetic acid to get acid soluble collagen. Collagen which is not soluble either in neutral salt or acid is termed as insoluble collagen.

Reagents :

1. 0.15 M NaCl : Dissolve 0.766 g of NaCl in water and make up the volume to 1 l with water.
2. 0.02 M sodium citrate, pH 3.5 : Dissolve 29.4 g of sodium citrate in 400 ml of water, adjust the pH to 3.5 and make up the volume to 500 ml with water.

Procedure :

Conduct all the experimental procedures at 4°C.

Kill the rat and immediately shave off the fur. Remove the skin, except from the head, tail and lower part of the legs. Immediately dissect adhering subcutaneous tissue and fat. Mince the hair free skin in a meat mincer.

Salt soluble collagen : Take 1 g of the freshly minced skin in a screw capped glass tube (50 ml capacity) and add 5 volumes of ice cold 0.15 M NaCl solution. Shake the tubes for 24 hr at 4°C. Centri-

fuge the samples at 20,000 g for 1 hr and decant the supernatant. Repeat the extraction procedure 4 times. Combine the supernatant solutions containing the soluble collagen and dialyse overnight against 20 volumes of distilled water at 4°C. Concentrate the nondialysable solution under vacuum at 40°C. Then hydrolyse 2 ml of the above solution with 6 N HCl at 110°C for 24 hr in a sealed ampoule. Estimate hydroxyproline in the hydrolysed samples (Refer hydroxyproline estimation).

Acid soluble collagen: To the residue remaining in the tube after extracting the salt soluble collagen add 6 ml of 0.2 M sodium citrate and shake the tubes at 4°C for 24 hr. Repeat the extraction procedure 3 times. Centrifuge and dialyse the samples in the same way as for the salt soluble collagen. Hydrolyse 1 ml of acid soluble collagen in 6 N HCl at 110°C for 24 hr in a sealed ampoule. Analyse the samples for hydroxyproline (Refer hydroxyproline estimation).

Insoluble collagen: Wash the residue remaining in the tube after extracting the soluble collagen with distilled water to remove sodium citrate, dry it at 60°C to a constant weight and defat it with a boiling mixture of ethanol and ether (2 : 1). Hydrolyse 10 mg of the fat free dried skin in 6 ml of 6 N HCl at 110°C for 24 hr in a sealed ampoule. Estimate hydroxyproline in the hydrolysate. Calculate collagen content on the assumption that rat collagen contains 13.4 % of hydroxyproline.

Adopt same procedure for the fractionation of collagen from other tissues also.

Reference:

J. Clin. Invest. **43**, 453 (1964).

10. AMINO ACIDS

a. Paper chromatographic procedure

Principle:

Free amino acids in a protein-free filtrate are separated by paper chromatography. The amino acid ninhydrin colour complex is converted to a deeply coloured copper complex and then evaluated densitometrically.

Reagents:

1. Ninhydrin reagent, 0. 25% in pure acetone.
2. N-butanol : aectic acid : water (12 : 3 : 5)
3. Copper nitrate solution : I ml of saturated copper nitrate and 0. 2 ml of 10% HNO_3 made upto 100 ml with pure acetone. (Acetone is preferable to alcohol, which was used in the original method).
4. Standard amino acid mixture.

Amino acid	mg/10 ml
Valine	28.8
Alanine	18.9
Lysine	16.7
Leucine	15.3
Tyrosine	12.4

Amino acid		mg/10 ml
Threonine	..	11. 3
Glycine	..	10. 7
Serine	..	10. 7
Histidine	..	9. 1
Taurine	..	9. 1
Isoleucine	..	9. 0
Tryptophan	..	8. 2
Phenylalanine	..	7. 6
Arginine	..	7. 7
Ornithine	..	6. 7
Citrulline	..	2. 5
Methionine	..	2. 5
Aspartic acid	..	0. 4
Cystine	..	1. 0
Glutamic acid	..	0. 8
Glutamine	..	20. 0

The mixture is dissolved by warming with a few drops of 6N HCl and some water It is diluted 100 times before spotting.

Procedure :

Processing of sample, Food stuffs : A homogeneous powder is made of foodstuffs. One g of this powder is extracted in 100 ml of 70% alcohol for about 8 hr (preferably overninght) with occasional stirring. The extract is filtered using Whatman No. 1 and the clear alcoholic extract is evaporated to dryness on a steam bath. If glutamine is to be identified, the extract is evaporated at room temperature using a current of air. The residue is then taken up in 10 ml of 10% isopropanol and filltered and the clear extract is used for spotting. This extract may be stored in small bottles at 4°C. Twenty μl of this extract is usually enough for ascending or descending chromatography. For a two way run, about 100 μl is needed for spotting.

Plasma/Serum/CSF : The protein free filtrate is used. This can be prepared by precipitating the proteins with 8 to 9 volumes of alcohol. To 1 ml of the material is added 9 ml of 80% ethanol, shaken well and centrifuged. The supernatant solution is evaporated to dryness and taken up in 1 ml of water or 10% isopropanol. This extract (100-500 μl) is used for spotting.

Urine : The sample is preserved by addition of thymol or preferably isopropyl alcohol containing thymol crystals (5 ml of a 10% solution of thymol in isopropanol/l). Desatlted samples of urine are used. The amount needed for spotting for a good separation has to be fixed by the investigator. However, the following guidelines have been suggested.

1. 50 μl of desaltad specimen or
2. a urine volume containing 250 μg of total N or
3. urine volume containing 0. 015 mg of creatinine.

Fifty μl of isopropanol extract corresponding to 50 μl or 100 μl of plasma is spotted on Whatma

No. 1 chromatographic paper over a width of 3 cm in closely spaced uniform spots forming a streak. Diluted standard amino acid mixture, 50 µl is also spotted.

The paper is irrigated with n-butanol-acetic acid-water solvent system (12 : 3 : 5) for 17 hr by the descending technique. The paper is air dried and sprayed with 0. 25% ninhydrin solution in acetone. The sprayed paper is air dried for 10 min and heated at 50°C for I hr and left overnight in the dark. Drying at low temperatures gives a clean background and the spots develop evenly. The papers are then sprayed with the copper nitrate solution. The copper complex colour is more intense and the chromatograms can be preserved for a long time with this reagent.

The intensity of the spots is evaluated by scanning in a densitometer at 509 nm.

Normally, the plasma amino acids get separated into 12 spots and these are identified with direct reference standard. It is essential that the spots should be uniform and straight, otherwise the results from scanning will be erroneous.

The area underneath each peak is determined and the density of the different spots is expressed as the per cent of the total density (ie. area under each peak divided by sum of areas under all peaks). When the total amino nitrogen concentration in the plasma is known the absolute concentrations of the individual groups can be calculated.

The above method is useful to get a gross separation of the amino acids and a semiquantitative estimation.

Reference :

Am. J. Clin. Nutr. **14**, 320 (1964).

b. Automatic amino acid analysis

Principle :

The food proteins are hydrolysed to free amino acids by hydrolysis with HCl. Carbohydrate rich foods tend to form a dark brown or black mass called humin during hydrolysis. This humin should be removed immediately from the hydrolysate to prevent destruction of amino acids in the latter.

During acid hydrolysis cystine and methionine in the sample can get partially destroyed and will thus be underestimated in the amino acid analyser. These two amino acids can be quantitatively converted to their oxidation products with performic acid oxidation which protects them from destruction. Cystine emerges as the cysteic acid peak and methionine as its sulfoxide or sulfone peaks on the chromatogram.

Reagents :

1. 6 N HCl
2. Diluent buffer : 0.2 N trisodium citrate buffer, pH 2.2
3. 30% H_2O_2
4. 85% formic acid
5. 48% HBr
6. Formic - performic acid mixture : Add 1 ml of 30% H_2O_2 to 9 ml of 85% formic acid. Let the mixture stand for 1 hr at room temperature. Cool to 0°C.

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TM-120

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Bangalore-560034
India

Procedure :

Weigh out accurately about 10 mg protein in a 10 ml ampoule. Add 5 ml of 6N HCl. Freeze the mixture in dry ice and attach the ampoule to a high vacuum pump to evacuate the air. Seal the neck of the ampoule after vacuum has been created. Hydrolyse the sample in a hot air oven at 110°C for 22 hr.

After the hydrolysis is over, break the seal of the ampoule. Remove the contents immediately by filtration through a nitrogen free filter paper (Whatman No. 1 paper previously washed with 1% HCl to remove the NH_3 and any ninhydrin positive substance). Wash the filter paper twice with distilled water. Make up the volume of the filtrate to 25 ml.

Take a 5 ml aliquot and flash evaporate it. Remove traces of HCl by adding about 2 ml distilled water to the residue, dissolve it and again flash evaporate. Repeat the washing procedure with distilled water. The last few drops might have to be evaporated in a vacuum desiccator over NaOH pellets.

Pipette 2.5 ml of pH 2.2 buffer into the flask and swirl to dissolve the contents. Store sample at 0°C.

Load 0.5 or 1.0 ml on each of the 2 columns of amino acid analyser for acidic and basic amino acid analysis respectively.

Weigh out 2—5 mg protein in a 10 ml ampoule. Add 2 ml performic acid solution to the ampoule. Let the reaction mixture stand at 0°C for 4 hr for soluble proteins or overnight for proteins that do not dissolve in the formic-performic acid mixture. Then add 0 3 ml of 48% HBr with swirling of the ampoule in an ice bath. Place the ampoule in a vacuum desiccator over NaOH pellets. Apply partial vacuum through a pump till the bromine distils over. Then apply full vacuum to evaporate the solution to dryness. Hydrolyse the sample at 110°C as described earlier, but only for 6 hr. Run cysteic acid, methionine sulfone and methionine sulfoxide standards along-side in the amino acid analyser.

Note :

For other biological materials, the supernatant after precipitation of the proteins with appropriate precipitant may be used.

The above procedure destroys tryptophan. For tryptophan analysis, alkaline hydrolysis followed by microbiological assay must be done.

References :

1. Instruction Manual Beckman Model 120 C Amino acid Analyser.
2. Methods in Enzymology, Vol. VI, p. 819 (1963).
3. J. Biol. Chem. **238**, 235 (1963).

c. Microbiological assay

Principle :

Refer Section A under microbiological assay.

Reagents :

1. Microorganism : *Leuconostoc mesenteroides* P—60 (A.T.C.C No. 8042).

2. Adenine Guanine-Uracil (AGU) solution : Adenine sulphate, guanine hydrochloride and uracil (0.2 g each) are dissolved in water using concentrated HCl to aid solution and the volume made upto 100 ml.
3. Xanthine : 0.2 g of xanthine is made upto 100 ml with water using ammonia for dissolving.
4. Salt A : K_2HPO_4 and KH_2PO_4, each 25.0 g are dissolved in 250 ml of water.
5. Salt B : The following salts are dissolved in water to make 250 ml of solution. A few drops of concentrated HCl are added to obtain a clear solution

$MgSO_4 . 7H_2O$	—	10.0 g
NaCl	—	0.5 g
$FeSO_4 . 7H_2O$	—	0.5 g
$MnSO_4 . 4H_2O$	—	0.5 g

6. Vitamin solution :

PABA	—	2 mg
Biotin	—	0.02 mg
Ca-pantothenate	—	10 mg
Folic acid	—	0.2 mg
Niacin	—	20 mg
Pyridoxal HCl	—	26 mg
Pyridoxamine 2 HCl	—	26 mg
Riboflavin*	—	10 mg
Thiamine	—	10 mg

Dissolved in water and made upto 100 ml.

*Riboflavin is weighed separately, dissolved in a few ml of acetic acid and mixed with other solution.

7. Non-essential amino acid solution :

DL—alanine	—	4 g
L—asparagine	—	8 g
L—aspartic acid	—	2 g
L—cystine	—	1 g
L—glutamic acid	—	6 g
Glycine	—	2 g
L—proline	—	2 g
DL—serine	—	1 g
L—tyrosine	—	2 g

These amino acids are dissolved in HCl and made upto 100 ml with water.

8. Essential amino acids :

L—arginine HCl	—	4.85 g
L—histidine HCl	—	1.24 g
DL—isoleucine	—	5 g
DL—leucine	—	5 g
DL—lysine HCl	—	5 g
DL—phenylalanine	—	2 g
DL—threonine	—	4 g
DL—tryptophan	—	0.8 g
DL—valine	—	5 g
DL—methionine	—	2 g

For dissolving, a few drops of conc. HCl may be used and then the volume made upto 100 ml. (Each amino acid solution to be prepared separately).

9. Basal medium :

Glucose	—	5 g
Sodium acetate 3 H_2O	—	4 g
Ammonium chloride	—	0.6 g
Salt solution A	—	1 ml
Salt solution B	—	1 ml
AGU solution	—	1 ml
Xanthine	—	1 ml
Vitamin solution	—	1 ml
Non-essential amino acids solution	—	1 ml
Essential amino acids (each essential amino acid to be added separately)	—	1 ml

The solution is mixed well, the pH adjusted to 6.8 and the volume made upto 100 ml.

(In the preparation of the basal medium that amino acid which is to be assayed should b omitted).

10. Indicator : 0.1 % Bromothymol blue.

Procedure :

Acid hydrolysis : To 1 g of the sample is added 25 ml of 2.5 N HCl. The mixture is then autoc laved for 6 hr at 15 lb pressure, cooled and 2 ml of 2.5 M sodium acetate is added. The pH is adjuste to 4.5 and the solution made upto a known volume and filtered. An aliquot is taken and the pH adjusted to 6.8 with NaOH and diluted to the required concentration.

Alkali hydrolysis (For tryptophan and tyrosine assays only) : To 1 g of sample is added 25 m of 2 N NaOH and proceeded as per acid hydrolysis. Racemisation takes place during this treatme and the amino acids will all be in DL form.

Preparation of stock standard solution : The amino acids that are used for the preparation standard solutions should be in the L-form. Fifty mg of the amino acid is dissolved in 100 ml to ma 500 μg/ml. A few drops of conc. HCl may be needed to dissolve some of the amino acids.

		*Dilution (to 50 ml) (ml)
Arginine	—	4.0
Histidine	—	1.0
Isoleucine	—	2.5
Leucine	—	2.5
Valine	—	2.5
Methionine	—	1.0
Phenylalanine	—	1.0
Threonine	—	2.0
Tryptophan	—	0.5
Lysine	—	4.0

*The dilutions are made from a stock standard solution containing 500 μg amino acid per ml.

Standard levels : 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0,7, 0.8 and 1.0 ml are taken in duplicates, enough water is added to make 1 ml followed by 1 ml of the basal medium.

Sample levels : 0.2, 0.4, 0.6, 0.8 and 1.0 ml are taken in duplicates and enough water is added to make to 1 ml followed by 1 ml of basal medium.

Sterilisation : The tubes are sterilised by autoclaving for 12 min at a pressure of 12 lb.

Incubation : For 72 hr at 37 C.

Titration : Against N/50 NaOH (0.02 N).

Assay of L-cystine and L-tyrosine :

1. H_2O_2 treated peptone : Fifty g of Difco Bacto Peptone is dissolved in 500 ml of 1 N HCl and 32 ml of 20 volume hydrogen peroxide is added. The mixture is allowed to stand overnight at room temperature, after which it is steamed in an autoclave for 30 min, cooled and the pH adjusted to 6.8 with 40 % NaOH. It is steamed again for 1 hr to remove the H_2O_2, cooled and diluted to 1 l and kept in the cold.

2. L-methionine — 0.5 g/50 ml
3. L cystine — 0.5 g/50 ml
4. DL-tryptophan — 1.0 g/50 ml
5. Glycine — 0.5 g/50 ml
6. L-tyrosine — 0.5 g/50 ml
7. Vitamin solution : The following are dissolved to make 100 ml.

Thiamine	— 10 mg
Pyridoxine	— 16 mg
Ca-pantothenate	— 10 mg
Riboflavin	— 20 mg
Niacin	— 20 mg
PABA	— 0.5 mg

8. Biotin — 1 µg/ml
9. Salt A, salt B, AGU and xanthine solutions are as described above.
10. Basal medium :

		in 100 ml
H_2O_2 treated peptone	—	15 ml
L-methionine	—	1 ml
L-cystine*	—	1 ml
L-tyrosine*	—	1 ml
DL-tryptophan	—	1 ml
Glycine	—	1 ml
Glucose	—	212 g
Sodium acetate 3 H O	—	2.2 g
Ammonium chloride	—	0.6 g
AGU solution	—	0.6 ml
Xanthine	—	0.6 ml
Biotin	—	0.5 ml

Vitamin solution	—	1 ml
Salt A	—	0.5 ml
Salt B	—	0.5 ml

The pH is adjusted to 6.8 and the volume made upto 100 ml.

*The amino acid to be assayed should be omitted from the basal medium.

Standard levels: As described earlier, cystine 0 to 35 μg/ml and tyrosine 0 to 50 μg/ml.

Sample levels: For cystine, the acid hydrolysed extract may be used. For tyrosine, the alkali hydrolysed extract may be used.

The samples are diluted after adjusting the pH to 6.8 to get a final concentration of 10 μg/ml. 0.5, 1,2 and 3 ml (in duplicate) levels are taken for assays, made to 5 ml with water and 5 ml basal medium added.

12. Sterilisation: 12 lb for 12 min.

Incubation: For cystine 18-24 hr are required for incubation and readings are taken turbidimetrically. For tyrosine 72 hr incubation period is necessary and titration is done against N/10 NaOH, using 0.1 % bromothymol blue as indicator.

Calculation:

The amino acid content in the unknown tubes is determined by interpolation of titer values on the standard curve. Then the amino acid content is calculated from the average of the values for 1 ml of the test solution.

$$\text{Amino acid concentration} = \frac{\mu\text{g per ml x dilution}}{\text{Wt of the sample}}$$

$$\text{For 100 g of protein} = \frac{\mu\text{g per ml x dilution}}{\text{Wt of the sample}} \times \frac{100}{\text{Protein (g)}}$$

References:

1. J. Biol. Chem. **177**, 533 (1949).
2. The microbiological assay of the vitamin B-complex and amino acids, E. C. Barton-Wright (Ed.) p. 142 (1952).

11. ALPHA AMINO NITROGEN

a. Naphthaquinone method

Principle:

The α – amino acid nitrogen is determined by photometric measurement of the colour produced when amino acids react with sodium β-naphthaquinone-4- sulfonate. Since the absorption curves of colours produced with different amino acids do not coincide, a mixed glycine-glutamic acid standard

is used as a reference because the colour formed closely matches to that obtained with blood filtrates. Standard versus reagent blank peak occurs at 470 nm. For plasma or blood, the reaction is applied directly to a protein free filtrate.

Reagents:

1. 10% (w/v) sodium tungstate.
2. 2/3 N H_2SO_4: Dilute 35.0 g concentrated H_2SO_4 to 1 l with distilled water, titrate for accuracy.
3. 0.5% (w/v) Sodium β-naphthaquinone-4 sulfonate in water, freshly prepared before use.
4. 2.0% (w/v) Sodium tetraborate (Borax) solution (stable indefinitely).
5. 0.1 N NaOH
6. 0.04 M Acid-formaldehyde reagent: 3 ml of 40% formaldehyde dissolved in 1 l of 0.3 N HCl.
7. 0.05 M Sodium thiosulfate: Dissolve 12.4 g $Na_2S_2O_3.5H_2O$/l of water.
8. Stock standard amino acid solution, (Store in refrigerator): Transfer 0.268 g glycine, 0.525 g glutamic acid, and 1.0 g sodium benzoate to a 500 ml volumetric flask. Add about 200 ml water and shake to dissolve the solids and add 4 ml conc. HCl, dilute to mark and mix. This contains 0.2 mg α-amino nitrogen/ml.
9. Working standards: Dilute 1.0 ml stock to 10.0 ml with distilled water (This contains 0.02 mg α-amino nitrogen/ml). Take 0.5, 1.0, 1.5, 2.0 and 3.0 ml solution and make to 5.0 ml with water to get 10, 20, 30, 40 and 60 μg amino nitrogen respectively.

Procedure:

Mix 1.0 ml plasma, 8.0 ml water, 0.5 ml 2/3 N H_2SO_4 and 0.5 ml 10% sodium tungstate and filter.

Set up in test tubes: (a) Reagent Blank-5.0 ml water. (b) Working Standard in 5.0 ml. (c) Test 5.0 ml of 1 : 10 plasma protein free filtrate.

Add one drop of phenolphthalein indicator to each tube, followed by 0.1 N NaOH drop by drop until permanent pink colour is obtained. A drop or two of alkali excess has no effect, but insufficient alkali (pH less than 9.2) as indicated by faint or transient pink colour will produce low results.

Add 1.0 ml borax solution and mix.

Add 1.0 ml naphthaquinone reagent, mix and immediately place in a boiling water bath for 10 min.

Place the tubes in cool tap water for 5 min, not longer.

Add 1.0 ml acid- formaldehyde reagent and mix. Add 1.0 ml sodium thiosulfate reagent and mix. Dilute the contents to 15.0 ml with water and mix.

Read absorbance against distilled water at 470 nm.

Reference:

Clnical chemistry, R. J. Henry (Ed) p. 315 (1964).

b. Fluorodinitrobenzene method

Principle:

Free amino groups of amino acids react with dinitrofluorobenzene (DNFB) in a basic medium

(pH 9-10) to form dinitrophenyl derivatives. These amino derivatives are extracted into acidified dioxane and the subsequent colour is read at 420 nm.

Since the absorption curves of colours produced with different amino acids do not coincide, a mixture of glycine and glutamic acid is used as a reference because the colour formed closely matches to that obtained with blood filtrates.

Reagents :

1. Stock DNFB : Dissolve 0.65 ml 2, 4-dinitrofluorobenzene in 50 ml acetone and store in a refrigerator. (Discard after 3 months. Caution should be exercised when pipetting DNFB solution and acidified dioxane reagent).
2. Sodium tetraborate solution (0.132 M) : Dissolve 50 g of $Na_2B_4O_7.10\,H_2O$ in water and dilute to 1 l.
3. Working DNFB solution : To l volume of stock DNFB add 9 vol of tetraborate solution immediately before use.
4. Conc. HCl in dioxane : Dilute 1 ml conc. HCl to 50 ml with dioxane.
5. Sodium tungstate (13.3%, w/v): Dissolve 133 g of sodium tungstate in water and dilute to 1 l.
6. HCl 0.11 N : Place 1 l of water in a 2 l volumetric flask. Slowly add 19 ml conc. HCl. Allow to cool to room temperature. Make up the volume with water.
7. Plasma protein precipitant : Mix 1 vol of 13.3% sodium tungstate solution with 9 vol of 0.11 N HCl. (This solution must be made on the day of use).
8. Stock amino acid solution (20 mg α-amino nitrogen /100 ml) : Dissolve 1.05 g of glutamic acid and 0.536 g glycine in 100 ml. Add 2.0 g sodium benzoate and 700 ml of 1 N HCl and dilute to 1 l. This contains 0.2 mg amino nitrogen per ml.
9. Working amino acid solution : Into separate test tubes measure 0.5, 1.0, 1.5 and 2.0 ml stock amino acid solution and make up the total volume in each to 5.0 ml with water. These represent 2, 4, 6 and 8 mg α-amino nitrogen per 100 ml equivalent of plasma.

Procedure :

Take into different tubes :

Blank –1.0 ml water, standards –0.5 ml of different standards + 0.5 ml water, unknown, –0.5 ml plasma + 0.5 ml water.

Add 4.0 ml of protein precipitant to each and mix well. Let the precipitation complete for 5 min.

Centrifuge the mixture for 10 min at 1500 g until the supernatant is clear and free of particles and decant the fluid carefully into another tube. instead, the mixture may be filtered to obtain clear filtrate. (For standards and blank, this step is not necessary).

Add 1.0 ml of working DNFB solution to 1.0 ml of supernatant fluid or filtrate and incubate at 70°C for 15 min. Remove the mixture from the incubation bath and let stand 5–10 min.

Add 5.0 ml of HCl in dioxane to the tubes containing the sample and reagent blank.

Read the absorbance of the sample against reagent blank at 420 nm in a suitable photometer to obtain the net absorbance.

Reference :

Standard methods of clinical chemistry, R.P. Mac Donald (Ed), Vol VI, p. 89 (1970).

c. Ninhydrin method

Principle :

When treated with ninhydrin, most amino acids are oxidatively deaminated. The resulting ammonia reacts with ninhydrin and its reduced product (hydrindantin) to give a blue substance diketohydrindylindene – diketohydrindamine, which has an absorption maximum at 570 nm.

Reagents :

1. 4 N sodium acetate buffer, pH 5.5. 540 g of NaOAc $3H_20$ is dissolved in about 400 ml water with heating if necessary. Cool solution and add 125 ml of glacial acetic acid and the volume made upto 1 l. The solution should have a pH 5.51. Final adjustment if needed is done by addition of NaOH. The buffer can be stored at 4°C without any preservative.

2. Ninhydrin reagent : Two grams of ninhydrin and 0.3 g of hydrindantin are dissolved in 75 ml of peroxide free methyl cellosolve. Stirring of the solution should be gentle so as not to incorporate air bubbles. Twenty five ml of the buffer at pH 5.5 are then added and the resulting reddish reagent solution is transferred immediately to a dark bottle. (The reagent is best prepared fresh every day).

3. 0.1 M citrate buffer, pH 5.0 : Prepare by dissolving 10.504 g of citric acid in 100 ml of I N NaOH diluted to 500 ml.

4. Diluent solution : Equal volumes of water and isopropanol are mixed.

5. Standard amino acid solution : Stock standard solution of leucine is prepared in 0.1 M citrate buffer, pH 5.0. Working standards are prepared (0.05 to 0 2 μmol/2ml) by dilution of the stock solution.

Procedure :

One ml of plasma or serum is taken in a test tube and 15 ml of chilled 95% ethyl alcohol is added with stirring. Leave in the cold for 15 min centrifuge and transfer the supernatant into an evaporating dish. The residue in the tube is washed with 2 ml of the chilled alcohol, stirred well centrifuged and the supernatant poured into the same evaporating dish. The alcohol is evaporated at a temperature below 60°C, to avoid cyclisation of glutamic acid. The evaporated residue is taken in I ml of 10% isopropyl alcohol in water and stored in the cold (– 4°C). The extract must be prepared as soon as possible and may then be stored for a week.

The isopropanol extract, 0.1 ml, is taken in tubes in duplicate and the volume made upto 2 0 ml with buffered citrate pH 5.0. To this is added 1.0 ml of the ninhydrin reagent. The tubes are stoppered, shaken briefly (less than 10 sec) and are heated for exactly 15 min in a boiling water bath. Five ml of the diluent solution is added, and the tubes transferred to a dry rack, cooled in cold water bath, and shaken thoroughly. The tubes should be shaken sufficiently in order to reduce the blank reddish colour of the hydrindantin to the desired level. The residual hydrindantin is oxidised by the air. After dilution the tubes are kept away from direct light and the intensity of the colour is read at 570 nm within $\frac{1}{2}$ hr after removal from the boiling water bath.

A standard curve is drawn with leucine using a similar procedure. The concentrations of amino nitrogen (in μ moles) in the unknown samples calculated using the standard graph.

Note :

If plasma is used, blood is collected with heparin as the anticoagulant.

In case of blood samples from undernourished children, the residue is more conveniently taken in 0.5 ml 10% isopropyl alcohol.

References :

1. J. Biol. Chem. **176,** 367 (1948).
2. J. Biol. Chem. **211,** 907 (1954).

12. TRYPTOPHAN

Principle :

The reaction involves the formation of the fluorophore, norharman from tryphophan by conden sation with formaldehyde and then oxidation with ferric chloride.

Reagents :

1. 75% TCA
2. 10% TCA
3. TCA/$FeCl_3$ solution : 10 g of TCA and 4.81 mg of $FeCl_3$ in 100 ml water.
4. HCl/$FeCl_3$ solution : 4.81 mg $FeCl_3$ in 100 ml of 0. 1mM HCl.
5. Formaldehyde (1.8%) : Dilute formalin which contains 40% formaldehyde appropriately with distilled water.
6. Standards : Tryptophan stock solution (250 nmoles per ml) is prepared by dissolving 5.1 mg of L-tryptophan in 100 ml of 0.IN ammonium hydroxide.

Norharman stock solution (250 nmoles per ml) is prepared by dissolving 4.2 mg of norharman (can be obtained from Aldrich Chemical Company) in 100 ml water containing 0. 25 g of TCA.

(All solutions are stable for 2 to 3 months. The stock solutions of tryptophan and norharman are stored at +4°C).

Procedure :

Plasma : Duplicate samples of plasma, 20 μl per sample are pipetted into 3 ml centrifuge tubes and 1.8 ml of TCA/ $FeCl_3$ is added and mixed and the mixture spun at 20,000 g for 10 min. The super-natant is decanted completely into a caliberated glass stoppered centrifuge tube (calibrated for 2 ml) and 0.2 ml of formaldehyde is added. After firmly stoppering the tubes are placed in a 99 to 101°C water bath for 1 hr. Following the reaction, the tubes are cooled to room tempeature and 10% TCA is used to replenish to the 2 ml mark the fluid lost during incubation. The product is read in a spectrofluorimeter at an excitation wavelength of 373 nm and an emission wavelength of 452 nm in a quartz or glass cuvette.

Liver : Fresh liver is weighed, immediately tansferred to a cold glass and teflon homogeniser and 7 parts of cold 0.9% saline are added and homogenised. Then 40 μl of homogenate is pipetted into a

plastic centrifuge tube, followed by 1.8 ml of $HCl/FeCl_3$. After thorough mixing, 0.2 ml of 75% TCA is added, the solution is again thoroughly mixed and spun at 20, 000 g for 10 min. All further steps are done with the supernatant as for plasma. Similar procedure can be used for the estimation of tryptophan in brain tissue also.

Urine : Fresh urine or urine stored at 9°C upto one week can be used. The procedure is essentially the same as the plasma method except that a final ethyl acetate extraction is used to concentrate the product and to remove high urine blank. If any turbidity is present after the addition of TCA, centrifugation is necessary as for plasma, before the addition of formaldehyde. After incubation, the mixture is made basic with 0.2 ml of 10N NaOH and extracted in the same tube (shaken by hand for 3 min) with equal volumes of ethyl acetate, 6 ml each. The ethyl acetate is then transferred with pasteur pipettes to similar tubes and the product is then reextracted from the ethyl acetate into 2 ml of 0.1N HCl and read as above.

Calculation :

All measurements are expressed as standard fluoroscence units, 5,000 fluoroscence units produced by 5 nmoles of non incubated norharman in 2 ml of 0.1N HCl. External and/or internal tryphophan standards are used for maximal accuracy.

Note :

1. The Farrand recording spectrofluorometer is equipped with the following : Corning No. 7-54 filter in the excitation monochromator. Corning No. 3.73 filter in the emitting monochromator. Calibration of the monochromator wavelength to $\pm$ 1 nm is done with a mercury arc. Slits used are 1 mm and 2 mm in excitation and emission monochromators respectively. Since excitation energy varies because of the instability of the xenon arc, a reference solution of norharman is read between samples.

2. Very low concentrations of molybdenum (approximately) 10^{-8} to 10^{-10} MoO_3) can totally inhibit the reaction. Temperature lower than 99°C during incubation seriously affect the yield; incubation at 94°C decreases the yield to 25%. After addition of $FeCl_3$ solution to the standards and samples they should be protected from sunlight and fluoroscent lights. It is better to carry out the further reactions away from light sources.

Reference :

J. Lab. Clin. Med. **69**, 160 (1967).

13. XANTHURENIC ACID AND KYNURENIC ACID

Principle :

Xanthurenic acid and kynurenic acid are separated on an ion exchange column and the fluorescence of xanthurenic acid is measured in alkaline condition whereas kynurenic acid is measured in acidic conditions.

Reagents :

1. Standard xanthurenic acid (XA), 500 μg/ml : 25 mg of xanthurenic acid is dissolved in 50 ml of 5% alcohol containing 0.1 ml concentrated NH_4OH.

2. Standard kynurenic acid (KA), 500 μg/ml : 25 mg of kynurenic acid is dissolved in 50 ml of distilled water with the addition of few drops of dilute NaOH.
3. Saturated NaOH solution.
4. HCl : 8N, 5N, 4N and 2N.
5. Ion exchange resin : Dowex-50 (H^+)-12% cross linkage is washed with water twice and 100 ml of this resin is packed in a column. This is washed with 2 l of 8 N HCl, 1.5 l of 4 N HCl and finally with 1 l of water.

Procedure :

Processing of sample : 100 ml of urine from a 24 hr urine collection is added to each of two measuring cylinders and diluted to 120 ml with water. To one of the cylinders 1 ml each of xanthurenic acid and kynurenic acid (500 μg each) standard solutions are added for recovery tests and 30 ml of 1 N HCl is then added to the two cylinders. The solutions are well mixed and loaded on the 3 cm long columns of Dowex-50. The columns are washed with 50 ml of 0.2 N HCl, 50 ml 0.5 N HCl and 20 ml water. The kynurenic acid and xanthurenic acid are then eluted with 400 ml of glass distilled water.

Xanthurenic acid : 1 ml standard solution (500 μg) is made upto 400 ml with distilled water. This contains 1.25 μg per ml of XA. Half, 1.0, 2.0, 3.0, 4.0 and 5.0 ml aliquots are taken and the volume in all tubes is made upto 5 ml by adding water. To this 5 ml of saturated NaOH is added. The tubes are well shaken and measured in EEL fluorimeter using OX 1 as the primary filter and 625 nm as the secondary filter.

Two and 4 ml aliquots of the eluates collected from the Dowex-50 (H^+) column are made upto 5 ml by adding distilled water. To this 5 ml of saturated NaOH is added and measured in EEL fluorimeter in the same way as the standard.

Kynurenic acid : One ml of standard solution (500 μg) is made upto 400 ml with distilled water. Half, 1.0, 2.0, 4.0 and 6.0 ml aliquots are taken in tubes and the volume in all is made upto 6 ml with distilled water. To this 4 ml of concentrated H_2SO_4 is added and mixed well, cooled and measured in the EEL fluorimeter using OX1 as primary filter and OB10 as secondary filter

Two and 4 ml aliquots of the eluate collected from Dowex-50 (H^+) are taken and the volume is made to 6 ml. To this 4 ml concentrated H_2SO_4 is added, cooled and measured in EEL fluorimeter.

Calculation :

$$\text{Xanthurenic or kynurenic acid in 24 hr urine (mg)} = \frac{X}{A} \times \frac{V}{1000} \times \frac{400}{100}$$

where V is the volume of the 24 hr urine, X is μg of XA or KA in the aliquot and A is the volume of the aliquot.

Reference :

J. Biol. Chem. **230**, 781 (1958).

14. TYROSINE AND ITS METABOLITES

Principle :

The Millon's reaction (colour with $HgSO_4$, H_2SO_4 and $NaNO_2$) is positive with tyrosine and its derivatives. Molar equivalents of tyrosine, p-hydroxyphenyl pyruvic acid and p-hydroxyphenyl acetic acid, all

ive rise to a similar orange red colour of about equal intensity. Since the phenols normally present in rine do not produce a red colour with Millon's reagent, this method permits an estimate of tyrosine and its vo metabolites. Since the colour is positive for both tyrosine and its derivatives, this method lacks specicity.

Reagents :

1. 15% $HgSO_4$ in 6 N H_2SO_4
2. 2 N H_2SO_4
3. 2% $NaNO_2$ in distilled water.

Procedure :

The 24 hr unine is collected in 10 ml of toluene and dilute HCl. To 2.5 ml of urine in a centrifuge ube is added 2 ml of 15% $HgSO_4$ and mixed well. The tube is allowed to stand for 1 hr, centrifuged for 5-20 min at 2000 rpm and the supernatant transferred into a test tube. The sides of the centrifuge tube re washed with distilled water and the washings are also transferred into the test tube. Then 10 ml of 2N $_2SO_4$ is added and the mixture heated in a boiling water bath for 15 min. It is then cooled and the ontents of the test tube are transferred into a 50 ml volumetric flask containing 10 ml 2N H_2SO_4. It is then lowed to stand for 30 min (from the time the tube is taken out from boiling water). If a slight cloudiness ppears, the tubes should be centrifuged again. Then 1 ml of 2% $NaNO_2$ is added to the flask, the volume ade upto 50 ml with distilled water and mixed. The intensity of the colour is read at once in a Klett-ummerson colorimeter at 470 nm with the blank adjusted to zero.

For blank, instead of urine, 2.5 ml of 2 N H_2SO_4 is taken in a test tube. Two ml of 15% $HgSO_4$ is dded and proceeded as above.

Standards are prepared by treating known amounts of tyrosine in 2 N H_2SO_4, in the same manner as e blank. For standards 125, 250, 500, 750, 1000 and 1250 μg of tyrosine concentrations are used.

Calculation :

Using the values for the standards, tyrosine and its derivatives in 2.5 ml of urine is determined and en calculated for the 24 hr collection.

References :

1. Biochem. J. **26**, 917 (1932).
2. J. Biol. Chem. **73**, 627 (1927).
3. Clinical Chemistry, Principles and Techniques, Harper and Row Publishers, R. J. Henry. (Ed) p. 344 (1964).

. HYDROXYPROLINE

Principle :

Hydroxyproline is oxidised to pyrrole-2 carboxylic acid, and then to pyrrole and the colour produced. hen p-dimethylaminobenzaldehyde is added, is measured at 560 nm.

Reagents :

1. Potassium borate, pH 8.7 : 61.84 g boric acid and 225 g KCl are dissolved in about 800 ml of distilled water. The pH is adjusted to 8.7 with KOH and the final volume is made upto 1 l. This buffer can be kept at room temperature.

2. Alanine solution : 10 g of alanine is dissolved in about 90 ml of distilled water, the pH is adjusted to 8.7 with KOH and the final volume is made up to 100 ml. The solution is stored in the cold with a thin layer of purified toluene to prevent mould formation.

3. Chloramine T solution : 0.2 solution in methyl cellosolve which is peroxide free is prepared daily just before use. Every time the compound is used, the lid of the container is sealed properly, kept in a desiccator over fused calcium chloride and stored in the cold.

4. Sodium thiosulphate, 3.6 M : Stored under toluene at room temperature.

5. p – Dimethylaminobezaldehyde (Ehrlich's Reagent) : Concentrated H_2SO_4 27.4 ml is slowly added to 200 ml of absolute alcohol in a beaker and the mixture is cooled. p–Dimethylaminobenzaldehyde 120 g is added to 200 ml of absolute alcohol in another beaker and then the acid-ethanol mixture is slowly added to the second beaker. The solution can be stored in the cold for several weeks; the crystals with precipitate on cooling are redissolved by warming the solution briefly.

6. Humin precipitant : Analytical grade anion exchange regsin (AG Cl^-X8 and 200-400 mesh) 20 g is mixed with 20 g of Norit A. The mixture is washed several times with 6 N HCl, ethanol and ether in a coarse sintered glass funnel and then dried.

7. Methyl cellosolve (peroxide free) : Methyl cellosolve is rendered peroxide free by shaking with ferrous sulphate keeping overnight, filtering and distilling. The distilled reagent is stored in dark bottles and kept in the cold. The peroxides in the reagent can be detected by acidification and addition of a solution of freshly prepared potassium iodide. A brown or yellow colour indicates the liberation of iodine which is due to the peroxide content. The iodine liberated can be detected by using starch indicator.

Procedure :

Determination of the total hydroxyproline in urine : More than 95% of the hydroxyproline in urine is present in the bound form. So, in order to estimate the total hydroxyproline, the urine is hydrolysed and the hydroxyproline determined.

Urine (0.1 – 1.0 ml) is placed in a tube which can be sealed for autoclaving. The volume is made up to 2 ml with water, and 2 ml of pure concentrated HCl is added. A maximum yield is obtained from urine by autoclaving at 124°C for 3 hr. For urine volumes greater than 1 ml a larger tube is used.

The hydrolysed sample is quantitatively transferred to a graduated centrifuge tube, and the volume is made upto 8.0 ml with water. The final HCl concentration should be 2 to 6 N. The rasin charcoal preparation is added with a spatula as necessary (usually about 1 g) and the solution is filtered through a quantitative filter paper into a graduated centrifuge tube. Seven ml of the filtrate is take in a graduated contrifuge tube which is previously standardised and to this is added a drop of 1% phenolphthalein. The pH ts adjusted to a faint pink colour by adding 10 N KOH first and then 0.1 N KOH and the volume is made upto 10 ml. The tube is cooled to room temperature.

To a 2.0 ml sample containing 2-10 μg of hydroxyproline in a screw capped test tube are added 2 g of solid KCl, 0.3 ml of a 10% alanine solution, pH 8.7 and 0.7 ml of 0.25 N borate buffer, pH 8.7.

exidation is carried out with 0.6 ml of freshly prepared solution of chloramine T in methyl cellosolve for exactly 20 min at room temperature. The reaction is stopped by adding 2.0 ml of 3.6 M sodium thiosulphate. The samples are again saturated with KCl and 3.3 ml of toluene is added. The tube is capped and shaken vigorously 100 times. The two phases usually can be separated by centrifuging briefly at low speed, but occasionally, it is necessary to break emulsions by agitating gently and recentrifuging. The toluene is discarded. If the presence of large amounts of interfering materials is suspected, the extraction is repeated. A second volume of 3.3 ml of toluene is added and the mixture allowed to stand in a boiling water bath for exactly 30 min. The tubes are cooled and then shaken vigorously 100 times and centrifuged briefly at low speed. The toluene layer (1.7 ml) is carefully removed by a dry pipette and transferred to a dry test tube. Even a tiny droplet of aqueous layer will affect the colour development drastically. To the toluene layer in the tube, is added 0.6 ml of paradimethylaminobenzaidehyde reagent with quick stirring. The developed colour is read after 15 min at 560 nm on a Beckman spectrophotometer against a blank treated in a similar manner (The colour is stable for about $\frac{1}{2}$ hr).

To estimate the free hydroxyproline in urine, the unhydrolysed urine is directly taken (2.0 ml), pH adjusted and processed as above.

The estimations should be carried out in triplicate and standards at two levels of concentration are run every time.

For a standard gragh, a solution (in water) containing 2 to 10 μg hydroxyproline/2 ml is treated in the same manner as described.

Calculation :

The concentration of hydroxyproline is calculated with reference to the standards and is expressed in terms of mg/24 hr.

The percentage of recovery of hydroxyproline is almost complete.

Note :

The procedure given for the preparation of Ehrlich's reagent is found to be unsatisfactory because exposing the reagent in the beaker during the preparation and warming the solution subsequently in order to redissolve the crystals formed during the cold storage, make the reagent acquire a very deep brown colour. With this type of reagent, the sensitivity of estimation is lost due to a very high blank reading. So it is advisable to prepare the solution in required amounts daily and use stoppered test tubes during the preperation to avoid exposure.

Twenty four hr collection of urine is made under toluene and the sample stored at 4 C.

References :

1. Anal. Biochem, **1**, 228 (1960).
2. J. Clin. Invest. **41**, 1028 (1962).
3. Ind. J. Med. Res. **54**, 848 (1966).
4. Lancet. **1**, 94 (1966).

16. AVAILABLE LYSINE IN FOODS

Principle :

The procedure involves conversion of lysine residues with reactive epsilon amino groups in the food proteins, into a yellow epsilon-dinitrophenyllysine by treatment of the material with fluorodinitrobenzene, followed by acid hyrolysis, Ether soluble interfering compounds are removed by extraction and

the extinction of the residual aqueous layer is measured. A blank value is obtained by treatment with methoxycarbonylchloride and extraction of the ether soluble lysine compound.

Reagents :

1. 10% NaOH.
2. Buffer, pH 8.5 : Mix 8 % $NaHCO_3$, 8 % Na_2CO_3, 19:1 (V/V) adjust pH with NaOH or HCl as the case may be.
3. HCl : 8.1 N, 1N
4. Fluorodinitrobenzene (FDNB) : 2.5% (V/V) solution in ethanol (to be prepared freshly).
5. Dinitrophenyllysine hydrochloride (DNPL-HCl. H_2O); Prepare a stock solution having 1 mg DNPL-HCl/ml in 1 N HCl. Working standard is made daily by diluting 1 ml of the stock standard solution to 100 ml in N HCl.

Procedure :

The material is first defatted and ground so as to pass through a 1/50 inch mesh and the nitrogen estimated by micro-Kjeldahl method.

The amount of the sample weighed should be such that it contains about 30-50 mg nitrogen. The sample is taken in a 250 ml round bottom flask to which is added 10 ml of 8% $NaHCO_3$ solution, the contents mixed by gentle swirling and then left to stand for 10 min. Add 0.3 ml of FDNB reagent to the flask. The contents of the flask are then thoroughly mixed and the flasks covered with black paper and shaken for 2 hr on a shaker. The alcohol is then removed by evaporation on a boiling water bath. The residue is extracted with four, 50 ml portions of anhydrous, peroxide free ether or till the ether washing is colourless. The ether each time is removed by decantation. The residual ether is removed by aeration. Add 24 ml of 8.1N HCl to the flask and the resulting mixture refluxed with gentle boiling for 16 hr. One or two glass beads are added to prevent bumping. The flasks are then ice cooled for 1–2 hr and filtered through a Whatman No. 41 filter paper in a 250 ml volumetric flask. The filtrate and washings are made up to volume with distilled water.

Portions of the filtrate, 2 ml are pipetted into two 10 ml glass stoppered test tubes marked A and B and also into a conical flask marked C. The contents of the tubes A and B are extracted twice with 5 ml of ether. The ether layer is discarded and the tube held in a boiling water bath until the effervescence ceases. The tubes are then allowed to cool to room temperature. Tube A is made upto the 10 ml mark with 1N HCl and kept for the final readings.

The contects of flask C are then titrated against 10% NaOH using phenolphthalein as the indicator and the contents of the flask discarded and the volume of NaOH used noted. The same volume of NaOH is then added to tube B, followed by 2 ml of buffer solution. Methoxycarbonylchloride (0 05 ml) is then added and the tubes shaken vigorously in order to disperse and dissolve the compound. (The extraction with methoxycarbonylchloride is done only to obtain a blank value for non-lysine interfering colour). After 5-10 min 0.75 ml of concentrated HCl is added, cautiously at first and later with agitation to prevent the contents from frothing.

The contents are then extracted twice with 50 ml portions of ether. The ether washing are discarded and the residual ether evaporated over a boiling water bath. The tubes are allowed to cool and then the volume made upto 10 ml with distilled water.

The OD of the contents of tubes A and B is measured in a photoelectric colorimeter at 420 nm.

Reading A minus B is taken as the extinction due to epsilon DNP-lysine, the concentration of which is extrapolated from the standard graph obtained by using concentrations of standard DNP-lysine solution.

Calculation :

Available lysine is expressed as g available lysine/100 g protein

$$\frac{0.851 \times 0.4682 \times \text{dil. factor} \times 100 \times 100 \times \text{conc. of DNP-HCl. } H_2O}{\text{Wt. of sample} \times \% \text{ protein}}$$

Mol. wt. of epsilon DNP-lysine = 312.3

Mol. wt. of epsilon DNP-lysine HCl. H_2O = 366.8

$\therefore$ Conversion of epsilon DNP-lysine HCl. H_2O to epsilon DNP-lysine, $= \frac{312.3}{366.8} = 0.851$

Conversion of epsilon DNP-lysine to lysine $= \frac{146.19}{312.3} = 0.4682$

Note :

It is not possible to check recovery by adding pure lysine at the beginning of the procedure, because, it is converted by FDNB at an alkaline pH into the ether soluble epsilon DNP lysine. Recoveries should, therefore, be measured from additions of 20-30 mg of epsilon DNP lysine after the addition of FDNB just before setting up the flask for refluxing.

Reference :

Biochem. J. **77**, 604 (1960).

17. CREATININE AND CREATINE

Principle :

Creatinine reacts with alkaline picrate to form a yellow-red colour known as the Jaffe reaction. Many substances present chiefly within the red cells contribute to a false high colour and therefore analysis of plasma or serum is preferable.

Reagents :

1. 10% sodium tungstate
2. 2/3 N H_2SO_4
3. Saturated picric acid solution : Distilled water is allowed to stand with an excess of picric acid and occasionally shaken well. The supernatant is decanted and used. If picric acid is not of chemically pure grade, it is recrystallised after dissolution in hot boiling glacial acetic acid.
4. Alkaline picrate reagent : Five ml of 10% NaOH are added to 25 ml of the saturated picric acid solution just before use.

Procedure :

Two ml of plasma or serum are added to 12.0 to 14.0 ml water in a test tube and mixed. Add 1 ml of 10% sodium tungstate solution and 1 ml of 2/3 N H_2SO_4. The solutions are mixed well and allowed to

stand for 10 min. The colour of the mixture turns brown. If the protein precipitation is complete. The mixture is filtered into a dry container.

When whole blood is used, 2 ml of blood is diluted with 14.0 ml water and 2 ml each of sodium tungstate and H_2SO_4.

Five ml of the tungstic acid filtrate are transferred to a test tube and 2.5 ml of the diluted picrate reagent is added, mixed and allowed to stand at 25°C for 15 min exactly and read in the colorimeter at 520 nm within the next 5 min. For best reproducibility, the time interval between the addition of the alkaline picrate reagent and the reading in the colorimeter should be kept constant for all tubes.

Working standard of creatinine solution containing 0.02 mg/ml is prepared. Various amounts of the solution containing 0.005 mg to 0.02 mg creatinine per tube are pipetted out, the volume made to 2.5 ml in all tubes. Water 7.5 ml and 5 0 ml of alkaline picrate reagent are added for development of colour. The rest of the procedure is the same as for samples.

A reagent blank with each set of samples and standards is run by heating 5 ml water with 2.5 ml picric acid and the OD of the reagent blank is deducted from that of the samples and standard to obtain the true values.

Determination of creatine : Five ml of the tungstic acid filtrate is transferred to a 25 ml graduated test tube. One ml of 1 N HCl is added, the test tube is covered with tin foil and heated in the autoclave at 130°C for 20 min. Five ml of freshly prepared alkaline picrate solution is added after the tubes cool down. The colour is developed for 10 min at 25°C and the volume made to 25 ml and read, observing the precautions as given under creatinine.

Standard curve is prepared with creatinine solutions containing 0.01 to 0.05 mg. The volume is all flasks made to 5 ml, 5 ml of acid and 5 ml of alkaline picrate reagent are added and the volume diluted to 25 ml after the colour is developed. Suitable correction is made for the reagents.

The performed creatinine as determined previously is deducted from the total creatinine value as obtained after autoclaving to get the creatine content expressed as creatinine.

Reference :

Physiological Chemistry, Hawk and Oser, (Eds.) 13th Edn. p. 555 (1954).

18. AMMONIA

Principle :

Ammonia and ammonium ion diffuse in the presence of alkali and are absorbed by boric acid in a Conway diffusion dish. This is titrated against acid.

Reagents :

1. Potassium carbonate 45%
2. Boric acid-indicator: 10 g of boric acid is added to 100 ml of water, to which is added 1 ml of 0.10% methyl red (in ethanol) and 8 ml of 0.1% bromocresol green (in ethanol). It is then diluted to 1 l and shaken until dissolved.

Procedure :

Into the outer chamber of a Conway diffusion dish is placed 1.0 ml of serum or whole blood in one pool. One ml of boric acid-indicator is placed in the central chamber. Lifting the cover, 1 ml potassium carbonate solution is placed in the outer chamber on the opposite side of the dish from the pool of serum. The cover is replaced and the potassium carbonate solution is mixed with the serum by totalling the dish on a flat surface. Diffusion is allowed to take place in the centre compartment for 20 min and the dish is placed on a magnetic stirrer. The solution is titrated rapidly with an ultramicroburette with N/1000 H_2SO_4.

Note :

Anticoagulant used in collection of blood may be heparin. Oxalates and sodium fluoride give fairly high values.

Reference :

Microtechniques of Clinical Chemistry, S. Natelson (Ed), 2nd Edn, p. 101 (1961).

19. UREA

a. Diffusion Method

Principle :

Urea is hydrolysed with urease and the ammonia so liberated is estimated.

Reagents :

1. Acetate buffer : Dissolve 18 g of anhydrous sodium acetate or 30 g of CH_3 COO Na, 3 H_2O in water, add 2 ml glacial acetic acid and dilute to 1 l.

2. Buffered urease solution : Hundred mg of urease powder is dissolved in 10 ml ot acetate buffer.

3. Other reagents are same as for ammonia determination except that 20 g of boric acid are dissolved and made to 1 l instead of 10 g.

Procedure :

Into the outer chamber of a Conway diffusion dish is added 0.1 ml of serum, blood or plasma followed by 0.5 ml of buffered urease solution. The mixture is kept in one pool on one side of the dish, covered and incubated at 45°C for 20 min. To the other side of the dish in the outer chamber is added 1 ml of the 45% K_2CO_3 solution. In the central chamber is now added 1 ml of the boric acid indicator solution, The solutions are mixed in the outer chamber by swirling the dish on a flat surface. The dish is kept again in the 45°C oven for 1 hr. Then it is placed over a magnetic stirrer and titrated in the center well with N/100 H_2SO_4.

Reference :

Microtechniques of Clinical Chemistry, S. Nateison (Ed), 2nd Edn. p. 440, (1961).

b. Aeration Method :

Principle :

Urea is converted to ammonia by treatment with urease. The mixture is then made alkaline with potassium carbonate solution and the liberated ammonia is absorbed into boric acid. The ammonium borate is then titrated with standard acid.

Reagents :

1. Phosphate Buffer, pH 6.8 : 6 g of KH_2PO_4 and 2 g of anhydrous NaH_2PO_4 in water are dissolved and diluted to 1 l and mixed. A few drops of chloroform is added as a preservative.

2. 10% urease solution : 1 g of urease is mixed in a little water, sufficient to make a paste and then water is added, a little at a time with stirring to make the final volume to 10 ml.

3. 2.0% Boric acid solulion with indicator : 20 g of boric acid are dissolved in about 500 ml of hot water, cooled. 2 ml of 1% bromocresol green is added. The mixture is diluted to 1 l and shaken.

4. Saturated pottassium carbonate : To 900 g of pure dry pottassium carbonate 1 l of water is added, stirred to dissolve as completely as possible.

5. 0.01 N H_2SO_4.

Procedure :

Two large test tubes are designated A and B. The tubes are fitted with tight fitting rubber corks with 2 holes bored. A long inlet tube dips into the solution inside the tube while the outlet tube is sufficiently high above the solution so that there is no chance for any solution passing over into the next tube. In tube A, the urea is acted upon by the urease. At the end of the incubation period, the ammonia is released by making the solution alkaline and immediately aeration is started to facilitate the carry over of ammonia. The air then bubbles through tube B which contains boric acid.

Take in tube A 2 ml of phosphate buffer and 5 drops of octyl alcohol and 0.5 ml of 10% urease solution. Add exactly 2 ml of blood mix gently, stopper loosely with a rubber stopper carrying the long inlet tube and a short outlet tube and allow to stand at 50-52°C temperature in an incubator or water bath for 10 min. During the time of standing prepare tube B by placing 25 ml of boric acid solution containing indicator and a drop of octyl alcohol and stopper tightly with rubber cork. At the end of the incubation period, connect the outlet of A tube to the inlet of B tube. Raise the rubber stopper of A tube and add 10 ml of saturated K_2CO_3 down the side of the tube. Immediately replace the stopper and tighten it firmly in place. Attach the long inlet tube of A by rubber tubing to a wash bottle half full of 1 N H_2SO_4 plus a few drops octyl alcohol and so arranged that when air is drawn through A it must first bubble through the wash bottle thus freeing it of any ammonia. Attach the short outlet tube of B by rubber tubing to a suitable source of suction (waterpump or vacuum line). When everything is ready turn on the suction carefully and draw air slowly through A and B for about 1 min, then increase the rate of air passage until the air is passing through as rapidly as possible. Continue the aeration for 1 hr.

At the end of the time slow down the rate of air passage (but do not stop it entirelv) to prevent any effect of back pressure and disconnect the tubes, startlng at the point from the source of suction.

Correction for any ammonia content of the reagents is made by running only reagents over the entire procedure.

Wash the tube B with little distilled water and titrate the contents against 0.01 N_2SO_4 to its original shade.

Calculation :

1 ml of 0.01N H_2SO_4 = 0.3 mg of urea.

mg of urea/100 ml of blood = Titer value – Blank value x 0.3 x $\frac{100}{2}$.

Reference :

Practical Physiological Chemistry, Hawk and Oser (Eds.) 13th Edn. p. 551 (1954).

c. Berthelot method

Principle :

The urea of plasma is split to ammonia and CO_2 by the action of urease and the ammonia is then determined photometrically by the phenol-hydochlorite reaction of Berthelot using sodium nitroprusside as a catalyst. The test is performed directly on the samples, there being no need to remove proteins because of the very high dilution used. The urease is buffered by EDTA which also performs the function of complexing metals that inhibit urease activity.

Reagents :

1. EDTA buffer: 1.0 g of disodium salt of EDTA is dissolved in 100 ml water and the pH is adjusted to 6.5.

2. Buffered urease solution : 15 mg urease (BDH) is suspended in 3.0 ml buffered EDTA. (Urease solution is stable for one month when kept refrigerated).

3. Phenol colour reagent : 50 g of phenol and 0.25 g sodium nitroprusside are dissolved in water and the volume made to 1 l. Stable for 2 months if kept in cold protected from light.

4. Alkali hypochlorite reagent : 25 g NaOH and 2.1 g sodium hypochlorite (40 ml of commercial bleach which contains 5.25 g NaOCl/100 ml can be used) are weighed and dissolved in 1 l of water. Stable at least for 2 months if kept in cold protected from light.

5. Stock urea standard: Place 0.429 g urea in one litre flask and make up the volume with water. Add a few drops of chloroform as preservative. Store in refrigerator. One ml of this contains 0.2 mg urea nitrogen.

6. Working urea standard : Take 1, 2, 3 and 4 ml of stock standard into separate tubes and add 3, 2, 1. and 0 ml water respectively to obtain standards of 5, 10, 15 and 20 mg urea nitrogen/100 ml.

Procedure :

1. Set up in test tubes :
   Blank — 20 μl water (this addition may be neglected)
   Standards — 20 μlof corresponding standard
   Unknown — 20 μl of plasma

2- The each tube odd 0.3 ml of ureese solution. mix and incubate for 30 min at 55°C, cool the tubes.

3. Add 1.0 ml phenol colour reagent to each tube, mix, then add 1.0 ml alkali-hypochlorite reagent and mix promptly again.

4. Incubate tubes again at 50 – 60°C for 5 min.

5. Add 8.0 ml of water to all the tubes.
6. Read absorbance values of standards and unknown against blank at 630 nm.

Calculation :

Since the treatments are the same for standards and unknown throughout the procedure, plasma values are obtained directlv from the stanard plot.

Urea in mg = Urea nitrogen in mg x 2.14.

Note :

It is necessary while setting up urea assay to be sure that one uses sufficient enzyme and proper conditions of incubation to obtain complete conversion of urea which may be present. This can be done as follows:

Prepare and run a standard ten times as concentrated as that of stock urea standard which contains 200 mg urea nitrogen/100 ml. The colour produced by the urease treatment should be the same as that obtained directly using 50 μl ammonia standard containing 200 mg ammonium nitrogen/100 ml (94.3 mg $(NH_4)_2 SO_4$/100 ml).

Reference :

Clinical Chemistry, Principles and Techniques. R. J. Henry, (Ed.) p. 266 (1964).

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III. CARBOHYDRATES

1. Glucose
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1. GLUCOSE

a. Glucose oxidase method

Principle:

Glucose is oxidised to gluconic acid by glucose oxidase. The hydrogen peroxide liberated is reduced by peroxidase and the oxygen transferred to an acceptor, which is colourlees in the reduced form but coloured in the oxidised form.

Reagents:

1. Protein precipitant:

Sodium tungstate, $Na_2WO_4 2H_2O$	—	10 g
Disodium phosphate NA_2HPO_4	—	10 g
Sodium chloride	—	9 g

Dissolve in about 800 ml water and add approximately 125 ml of 1 N HCl to adjust to pH 3.0. Add 1 g of phenol and make up to 1 l with water. Stable for 1 year at 25°C.

2. Colour reagent:

Sodium azide	—	0.3 g
4 – aminophenazone	—	0.1 g
Disodium phosphate	—	3.0 g

Dissolve in 295 ml of water, then add 5 ml Fermoozyme 952 DM. Stable for 8 weeks at 4°C.

3. Standard: Prepare a saturated solution of benzoic acid, by dissolving 1 g of benzoic acid in about 300 ml of hot distilled water. Filter whilst hot, through a coarse filter paper. Needle like crystals should form on cooling. Immediately before use, filter to remove crystals and use the solution for making up the standards.

The stock standard contains exactly 1g of pure glucose in 100 ml benzoic acid solution. The working standard is made by diluting 1 ml of stock standard with 49 ml with benzoic acid solution. Stable at 25°C for 1 year.

Procedure :

Pipette 0.1 ml of blood into 2.9 ml or protein precipitant. Mix well and centrifuge for about 5 min. A standard curve is set up for each batch of determinations. Into clean tubes pipette 0.1, 02 and 0.3 ml of the working glucose standards equivalent to 60, 120 and 180 mg per 100 ml. In each case make up the volume to 1 ml with protein precipitant reagent.

Similarly, take 1 ml of clear supernatant from the test and place in clean tubes. For the reagent blank use 1 ml of protein precipitant. To all tubes add 3 ml of colour reagent and incubate at 37°C for 10 min. Then place the tubes in cold water for 1 min and read the absorbance at 505 nm against the reagent blank without further delay.

Plot the absorbance of the standards graphically and read off the glucose values of the tests from this. If the glucose concentration of the test is greater than 180 mg/100 ml repeat the colour development stage using a smaller aliquot of the supernatant, e.g. 0.2 or 0 5 ml. Include additional standards in the calibration where necessary and multiply the result obtained by the appropriate factor, depending on the volume of supernatant used.

Note :

Fermoozyme 952 DM (Hughes and Hughes Ltd.) contains both glucose oxidase and peroxidase.

The chromogen, 4-aminophenazone replaces 0-tolidine and dianisidine, which are thought to be carcinogenic.

Reference :

J. Clin. Path. 22, 246 (1969).

b. Ferricyanide method

Principle :

Sugar is oxidised with alkaline potassium ferricyanide and the ferrocyanide produced is measured photometrically after the conversion.

Reagents :

1. Ferricyanide solution : Dissolve 0.5 g potassium ferricyanide in 1 l of distilled water. Store in brown bottle.
2. Carbonate-cyanide reagent : Dissolve 5.3 g sodium carbonate and 0.65 g potassium cyanide in 1 l of distilled water.
3. Ferric ammonium sulfate solution : 1.5 g ferric ammonium sulfate in 1 l of 0.05 N H_2SO_4.
4. Tungstate solution : 10% sodium tungstate.
5. Standard glucose solution : 90 mg glucose in 100 ml of saturated benzoic acid solution.
6. $N/12_2H_2SO_4$.

Procedure :

Blood from a haemoglobin pipette (0.02 ml) is added to 0.2 ml of N/12 H_2SO_4 and 8.78 ml of distilled water is added and mixed thoroughly. Sodium tungstate 0.02 ml is now added and the solution is mixed with a glass rod to precipitate the proteins. This is allowed to stand for 10 min and centrifuged. One ml of the above supernatant is mixed with 1 ml of ferricyanide solution and 1 ml of cyanide-carbonate solution. The solution is heated in a boiling water bath for 15 min and cooled immediately under running tap water. Now 5 ml of ferric ammonium sulfate (60 ml ferric ammonium sulfate + 0.12 ml tween 80) is added. The solution is mixed and readings taken at 690 nm in a Coleman spectrophotometre after 10 min and a within 30 min. The standard glucose is diluted as per the range required and treated as above.

Calculation :

A standard graph is drawn and the value for the serum sample is obtained from it (say X). This value multiplied by the dilution factor gives the glucose value in the sample.

0.02 ml blood is diluted to 9 ml

$\therefore$ 100 ml is diluted to $\frac{100 \times 9}{0.02} = 45000$

μg glucose/100 ml blood $= 45000 \times X$

mg glucose/100 ml blood $= \frac{45000}{1000} \times X = 45 \times X$

Reference :

Methods in Enzymology, Vol III, p. 86 (1957).

c. Hagedorn and Jensen method

Principle :

Blood proteins are precipitated with zinc hydroxide. The reducing sugars in the protein-free filtrate reduce potassium ferricyanide on heating. The amount of unreduced ferricyanide is determined iodimetrically.

Reagents :

1. 0.45% Zinc sulphate : Prepared fresh every week by dilution of 45% stock solution.
2. 0.1 N NaOH : Prepared fresh every week by dilution of 2 N NaOH.
3. Potassium ferricyanide solution : 1.65 g recrystallised potassium ferricyanide and 10.6 g anhydrous sodium carbonate are dissolved in 1 l water and stored in a dark bottle, protected from light.
4. Iodide-sulphate-chloride solution : Zinc sulphate 10 g and NaCl 50 g are dissolved in 200 ml water. On the day of use 5 g potassium iodide is added for 200 ml.
5. 3% Acetic acid
6. 0.005 N Sodium thiosulphate : Prepared fresh daily by the dilution of 0.5 N sodium thiosulphate. 0.5 N sodium thiosulphate is prepared by dissolving 70 g of the salt in 500 ml water. It is better if a solution of a slightly higher normality is prepared, since sodium thiosulphate decomposes soon. The solution should be protected from light and stored in the cold. The normality is checked daily with 0.005 N potassium iodate..

7. 0.005 N Potassium iodate : This is a stable solution and should be made accurately. 0.3566 g of the anhydrous salt is weighed accurately and dissolved in 2 l water. This is used to check sodium thiosulphate and potassium ferricyanide solutions.

8. Starch indicator : 1 g soluble starch is dissolved in 100 ml saturated NaCl solution.

Procedure :

One ml 0.1 N NaOH and 5 ml 0.45% zinc sulphate are pipetted into a test tube. Blood, 0.1 ml is taken in a dry micro-pipette and introduced into the gelatinous zinc hydroxide in the test tube rinsing out the pipette twice with the mixture. The tube is kept in a boiling water bath for 3 min and cooled without disturbing the precipitate. The mixture is filtered through a Whatman No. 42 filter paper of lightly pressed moistened cotton. The tube is washed twice with 3 ml portions of water and filter into the same containers.

Two ml potassium ferricyanide is then added to the filterate and heated in a boiling water bath for 15 min. After cooling 3 ml iodide-sulphate-chloride solution followed by 2 ml 3% acetic acid are added.

The liberated iodine is then titrated against 0.005 N sodium thiosulphate, using 2–3 drops of 1% starch as indicator towards the end of the titration.

A blank is run through the entire procedure simultaneously. The blank should give a titre value of 1.97 to 2.00 ml.

Calculation :

The value may be expressed as mg glucose/100 ml by consulting the table that is given in the Appendix. The value for blank is subtracted from the one for the unknown.

mg glucose/100 ml $= 0.385 \times \frac{X}{2.0} \times 1000$, where $X = 2.0 - B$ (titer value of sample) in terms of exactly N/200 thiosulphate.

Note :

Blood is collected into bottles containing sodium fluoride 10 mg/ml blood. The blood should preferably be processed immediately after collection and the protein free filterate may be preserved in the cold.

It is essential that potassium ferricyanide be recrystallised. The recrystallisation procedure is as follows :

The crude crystals are washed with cold water and then dissolved in hot water. The solution is filtered while hot, through a filter paper previously washed with hot water. The filtrate is allowed to crystallise in a dish surrounded by ice. The crystals are dried in an oven at 50°C and stored in a dark bottle.

Reference :

Practical Physiological Chemistry, Howk, Oser and Summerson Eds. 13th (Edn.) p. 577 (1954).

d. Nelson and Somogyl method

Principle :

Blood proteins are precipitated by zinc hydroxide. The filtrate is heated with alkaline copper eagent and the reduced copper formed is treated with arsenomolybdate reagent resulting in the formation of violet colour which is read in the photometer.

Reagents :

1. 5% zinc sulphate solution.
2. 0. 3 N Barium hydroxide:

These two solutions should be so adjusted that 5 ml zinc sulphate require 4. 7 to 4. 8 ml barium Hydroxide for complete neutralisation.

3. Alkaline copper reagent :

Solution A : 25 g anhydrous Na_2CO_3, 25 g Rochelle salt, 20 g Na_2HCO_3 and 200g anhydrous Na_2SO_4 are dissolved in about 800 ml water and diluted to 1 l. The solution is stored at room temperature and never below 20°C. It is filtered before use if any sediment is formed.

Solution B : 15% $CuSO_45H_2O$ containing one or two drops of concentrated H_2SO_4.

On the day of use 25 parts of solution A and 1 part of B are mixed. This is the alkaline copper reagent.

4. Arsenomolybdate colour reagent : Ammoniummolybdate 25 g is dissolved in 450 ml water 21 ml concentrated H_2SO_4 is added and mixed. Disodium orthoarsenate ($Na_2H\ AsO_47H_2O$) 3.0 g is dissolved in 25 ml water and is added with stirring to the acidified molybdate solution. It is then placed in an incubator at 37°C for 24-48 hr and stored in a glass stoppered brown coloured botile.

5. Standard glucose solutions (Stock glucose solution) : Exactly 1.0 g of anydrous pure glucose is dissolved in 10–15 ml 0.2% benzoic acid and diluted to 100 ml with benzoic acid solution.

Three working standards are prepared by diluting 0. 5, 1.0 and 2.0 ml of the stock solution to 100 ml with benzonic acid. These solutions in benzoic acid keep indefinitely at room temperature.

Procedure :

Into a test tube containing 3.5 ml of water is introduced through a clean dry micropipette, 0.1 ml blood and mixed well. To the tube is added 0.2 ml of 0.3 N barium hydroxide. After the mixture turns brown 0.2 ml zinc sulphate is added and mixed. After 10–15 min the mixture is filtered through a Whatman No. 1 filter paper.

Into 2 separate test tubes is transferred 1 ml aliquot of the filtrate, One ml alkaline copper reagent is then added. The tubes are covered with a glass marble and placed in a boiling water bath for 20 min. The tubes are then cooled under running water. One ml arsenomolybdate reagent is added and the solution diluted to 25 ml with water. Simultaneously standard and a reagent blank are similarly prepared.

The intensity of colour produced is read at 500 nm. The colour is stable and readings may be taken at convenience.

Calculation :

$$\text{mg glucose/100 ml} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{conc. of standard} \times 4 \times \frac{100}{0.1} \times \frac{1}{100}$$

Note:

Collection and preservation of blood is the same as described in the previous procedure. When the glucose concentration in the blood is low as much as 3 ml of the filtrate may be taken.

Reference:

Methods in Enzymology, Vol. III, p. 87 (1957)

2. LIVER GLYCOGEN

Principle:

Glycogen is hydrolysed to glucose and the glucose thus formed is estimated by any standard method.

Reagents:

1. 30% KOH
2. 95% Ethanol, 60 Ethanol
3. 2 N H_2SO_4

Procedure:

1. The liver is taken out rapidly from the animal and the excess blood removed by blotting between folds of filter paper and immediately put into a weighed stoppered test tube containing 30% KOH and weighed again. The amount of alkali is then adjusted to get 2 ml per g of liver.
2. The tissue is digested in a boiling water bath for $1\frac{1}{2}$ hr.
3. Cool in ice cold water. Two volumes of 95% ethanol are then added and the mixture heated just to boiling. Spurting is avoided.
4. It is left to stand overnight in the cold, cenrifuged, the precipitate is dissolved in 5-10 ml warm water. The glycogen is reprecipitated with 2 volumes of 95% ethanol.
5. The precipitate is centrifuged and washed several times with 60% ethanol.
6. Two ml of 2 N H_2SO_4 per g of liver is added and hydrolysed in a boiling water bath for 3-4 hr.
7. The solution is neutralised with NaOH using phenol red as indicator, then made to volume and filtered.
8. Glucose is determined in an aliquot.
9. The factor 0.93 is used to convert glucose to glycogen.

Reference:

J. Biol. Chem. **135**, 511 (1940).

3. STRACH

Principle:

Finely powdered cereals and pulses are treated repeatedly with hot 80% alcohol. The residue

rich in starch is solubilised with perchloric acid. Filtered perchloric acid extract is treated with anthrone-sulphuric acid to determine glucose.

Reagents :

1. Glucose standard : Dissolve 0.1 g of anhydrous glucose in 100 ml of water containing 0.1% benzoate. Dilute 10 ml of stock solution to 1 l and use 5 ml for the standardisation. Prepare the diluted standard fresh.

2. Anthrone sulphuric acid : Dissolve 2 g anthrone in 1 l of cold 95% H_2SO_4. Store at 4°C and prepare fresh every 2 days.

3. 80% Ethyl alcohol-water : Dilute 1.68 l of 95% ethyl alcohol to 2 l with water.

4. 52% Perchloric acid : Add 270 ml of 72% perchloric acid to 100 ml of water. Store in glass stoppered container.

Procedure :

Extraction of sugars and starch : Weigh 0.2 g of flour into a 50 ml centrifuge tube. Add a few drops of 80% alcohol to wet the flour and prevent clumping, add 5 ml of water and stir thoroughly. Add 25 ml of hot 80% ethyl alcohol, mix well and centrifuge after 5 min of standing. Decant and discard the alcoholic solution. Add 30 ml of fresh hot 80% ethyl alcohol, stir and centrifuge as before. Discard the alcoholic solution. Repeat this washing twice for a total of 4 washings or until a test with anthrone is negative.

To the residue after final centrifugation add 5 ml of water. Cool in ice water and while stirring add 6.5 ml of diluted perchloric acid reagent. Stir for about 5 min with a glass rod. Keep for 15 min stirring occasionally. Add 20 ml of water and centrifuge. Pour the aqueous starch solution into a 100 ml volumetric flask cooled in ice watar and stir while adding 6.5 ml of diluted perchloric acid reagent. Solubilise as before for 30 min at 0°C with occasional stirring and wash the contents of the tube into a 100 ml flask containing the first extract. Dilute the combined solutions to 100 ml and filter.

Determination of starch : Dilute 5 to 10 ml of the filtered starch solution to 500 ml or to contain, 25 to 100 μg of starch per 5 ml of solution. Pipette 5 ml of the diluted solution into a test tube, cool in a water bath and add 10 ml fresh anthrone reagent. After the anthrone reagent has been added to all the tubes, mix each one thoroughly and heat them together for 7.5 min. Read the colour in a photocolorimeter at 630 nm.

Prepare a standard curve every time using 0.50 and 100 μg glucose containing the same amount of perchloric acid as that in starch aliquots and use this calibrated curve to obtain the yield of glucose from starch. Multiply glucose value by 0.9 to convert into starch value.

Reference :

Anal. Chem. **22**, 1156 (1950).

4. α - AMYLOLYSIS *IN VITRO*

Principle :

Amylase splits starch into maltodextrins and glucose. Maltose and glucose form major products

of α -amylolysis. This method has been adopted to determine rate of α-amylolysis of starch present in cereals and pulses.

Reagents:

1. Pancreatic α-amylase.
2. 0.02 M glycerophosphate – HCl buffer pH 6.9
3. Alkaline salicylate reagent : 20 g of 3, 5-dinitro salicylic acid are suspended in approximately 400 ml of water. A solution of 32 g of NaOH in 300 ml of water is added dropwise while stirring and gently heated on water bath (if necessary) until a clear solution is obtained. 600 g of potassium sodium tartarate are added in small portions and water is added to final volume of 2 l. The reagent is stored in dark at room temperature,
4. starch 1% suspension in glycerophosphate buffer
5. Maltose: 2 mg/ml

Procedure:

One percent suspension (with reference to starch content) of test material in 0.02 M glycerophosphate-HCl buffer pH 6.9 prepared after thorough homogenisation. This material is used as substrate for α-amyloysis. The reaction mixture containing 0.01 mg of α-amylase suspended in 1 ml of 0.02 M glycerophosphate buffer pH 6.9 and 1.0 ml of the substrate are taken in individual test tubes and incubated in a metabolic shaker at 37°C. The reactions in different test tubes are stopped at different intervals (ie. 0, 3, 6 and 9 min) by adding 1.0 ml of alkaline salicylate reagent. The mixture is heated in a boiling water bath for 5 min, cooled and diluted to a total volume of 10 ml with water. The absorbance at 540 nm is measured in Coleman spectrophotometer. The *in vitro* α – amylolysis thus determined, gives an idea of the rate of starch digestion and can also be applied to cooked materials. One unit of α - amylase activity is expressed as the reducing sugar value equivalent to 1 mg maltose released per mg of the enzyme in 3 min. The calibration curve with standard maltose solution with a range of 0 to 2 mg is established using the alkaline salicylate reagent.

Reference:

Biochemical Preparations. E.H. Fischer and E. A. Stein (Edn.)Vol. 8, p. 30 (1961).

9. ANAEROBIC GLYCOLYSIS

Principle:

The Warburg manometric technique with the use of standard Warburg apparatus is adopted. The anaerobic respiration is studied in an atmosphere of nitrogen in the presence of fructose 1,6-diphosphate.

Reagents:

1. Na-K Phosphate buffer—0.03 M pH 7.3
2. Sodium bicarbonate—0.36 M
3. Magnesium sulphate—0.24 M
4. ATP — 0.002 M
5. NAD — 0.005 M
6. Nicotinamide — 0.6 M
7. Fructose 1.6 – diphosphate - 0.0075 M

Procedure :

The reaction mixture in the flask should contain 1.0 ml Na-K phosphate buffer, pH 7.3; 0.2 ml $NaHCO_3$; 0.1 ml each of ATP, NAD and nicotinamide solutions and 0.2 ml of fructose 1,6-diphosphate. Liver homogenate containing 50 mg fresh tissue (from 5% homogenate in phosphate buffer) is taken into the side arm of the flask. The total volume in the main chamber of the flask is made upto 3.0 ml. Temperature equilibration is obtained by shaking the reaction mixture for 10 min before the liver homogenate is tipped into the chamber from the side arm. Liberation of CO is measured by taking the reading, every 10 min for half an hr. Enzyme activity is expressed as μl CO_2 liberated mg nitrogen/hr.

Reference :

J. Biol. Chem. **161**, 197 (1945).

6. MEASUREMENT OF ENDOGENOUS RESPIRATION

Principle :

The oxygen uptake by the liver is measured by the Warburg constant volume manometric method.

Reagents :

1. Krebs-Ringer phosphate buffer, pH 7.4 : (Refer Appendix).
2. Potassium hydroxide, 10%.

Procedure :

In the main chamber of the Warburg flask is added 2 ml of Krebs-Ringer phosphate buffer and 1 ml of liver homogenate (10%). Into the central well, 0.2 ml of 10% KOH is taken. Respiration is allowed for 30 min at 37 C, using air as the gas phase. Readings are taken at 10 min intervals, which should be linear with time. Respiratory activity is expressed as μl O_2 uptake/mg nitrogen/hr.

References :

1. Ind. J. Med. Res. 53, 1087 (1965),
2. Manometric Techniques, Umbreit, W.W., Burris. R. H., Stauffer, J.F. (Eds.) p. 16 (1964)

IV. LIPIDS

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1. PHYSICO-CHEMICAL PROPERTIES

a. Specific gravity of fats and oils

The oil or fat sample may be preserved under an atmosphere of nitrogen in the cold. The sample should be allowed to reach the room temperature and mixed well before being subjected to the various analytical tests.

The specific gravity of a liquid is the weight of a given volume of liquid at the specified temperature, compared with the weight of an equal volume of water at the same temperature, all weighings being taken in air.

The specific gravity bottle (or pycnometer) is weighed empty (W_B).

The bottle is then filled completely with the liquid and weighed (W_L).

After cleaning, the bottle is filled completely with distilled water and weighed (W_w).

The temperature of the liquid is noted.

$$\text{Specific gravity} = \frac{W_L - W_B}{W_w - W_B}$$

The temperature at which the specific gravity is determined should be specified.

Reference :

British Pharmacopoeia Appendix IV-E, p. 1017 (1963).

b. Refractive index

The refractive index (n) of a substance is the ratio of the velocity of light in vacuum to its velocity in the substance. It varies with the wavelength of light used in its measurements. It may also be defined as the ratio of the sine of angle of incidence to the sine of the angle of refraction.

Clean the refractometer with alcohol and ether.

A drop of oil or fat (in case of a solid fat the temperature should be suitably adjusted by circulating hot water) is placed on the prism. The prism is closed by the ground glass-half of the instrument. The dispersion screw is adjusted so that no colour line appears between the dark and illuminated halves. The dark line is adjusted exactly on the cross wires and the refractive index is read on the scale.

Usually commercial instruments are constructed for use with white light but are calibrated to give the refractive index in terms of sodium light of wavelength, 589.3 nm at a temperature of 20 C unless otherwise specified.

Reference :

British Pharmacopoeia Appendix IV-B, p. 1016 (1963).

c. Acid value

The acid value of a fat is the number of mg of KOH required to neutralise the free acid in 1 g of the substance.

Reagents :

1. A mixture of equal volume of alcohol (95%) and ether
2. 1% phenolphthalein in alcohol
3. 0.1 N KOH

Procedure :

About 10 g of the oil or fat is weighed, accurately into a 250 ml conical flask to which is added 50 ml of a mixture of equal volumes of alcohol and ether previously neutralised after the addition of 1 ml of phenolphthalien solution. If necessary, the contents may be warmed in a water bath until the substance has completely dissolved. The solution is titrated with N/10 KOH with constant shaking until a pink colour persists for 15 sec. The titre value in ml (a) is noted.

$$\text{Acid value} = \frac{a \times 0.00561 \times 1000}{\text{Wt in g of substance}}$$

If the normality of KOH is not exactly 0.1 N and if it is N then the above equation is multiplied by the factor $\frac{N}{0.1}$.

Reference :

British Pharmacopoeia Appendix X-A, p. 1055 (1963).

d. Saponification value

Reagents :

1. KOH solution : 35 to 40 g of KOH pellets are dissolved in 20 ml of water to which is added sufficient alcohol (95%) to make 1 l. The solution is allowed to stand overnight and the clear supernatant is used for the estimation.
2. 0.5 N HCl
3 1% Phenolphthalien solution in 95% alcohol

Procedure :

About 2 g of the substance is weighed accurately in a 250 ml round bottom flask. Twenty five ml of the alcoholic KOH solution is added, a reflux air condenser is attached and the flask kept in a boiling water bath for 1 hr. The contents of the flask are frequently mixed. While the solution is still hot, 1 ml of phenolphthalein solution is added and the excess alkali titrated against 0.5 N HCl (a). The experiment is repeated without the oil or fat to obtain the blank value (b). Since 1 ml of 0.5 N HCl is equivalent to 0.02805 g of KOH, the following equation is used to calculate the saponification value.

$$\text{Saponification value} = \frac{(b-a) \times 0.02805 \times 1000}{\text{Wt in g of substance}}$$

If the HCl has a normality of 'N' then the above equation is multiplied by a factor of $\frac{N}{0.5}$.

Reference :

British Pharmacopoeia Appendix X-C, p. 1057 (1963).

e. Iodine value

The iodine value of a substance is the weight of iodine absorbed by 100 parts by weight of the substance.

Reagents :

1. Iodine monochloride solution : 5 ml iodine monochloride is dissolved in 500 ml glacial acetic acid

If iodine monochloride is not readily available, the solution may be prepared as follows : Iodine trichloride, 8 g, is dissolved in about 200 ml of glacial acetic acid with heating in a water-bath under moisture free condition (stoppered with a tube containing fused calcium chloride). Into another flask is taken 9 g of iodine and dissolved in 300 ml of carbon tetrachloride. The two solutions are mixed and sufficient glacial acetic acid is added to make the valume 1 l.

2. 0.1 N sodium thiosulphate solution
3. 15% KI solution
4. Starch mucilage

Procedure :

The substance is accurately weighed into a dry iodine flask and is dissolved by the addition of 10 ml of CCl_4 (or $CHCl_3$). Twenty ml of iodine monochloride solution is then added, the flask is stoppered with a stopper previously moistened with KI solution and the mixture allowed to stand in the dark for 30 min at a temperature between 15-25°C. Fifteen ml of KI solution is pipetted into the cup top,

the stopper is carefully removed and rinsed along with the sides of the flask with 100 ml of water, the flask is then shaken and the contents are titrated with 0.1 N $Na_2S_2O_3$ solution using starch mucilage as indicator towards the end of the titration (starch is added only when the colour of the reaction mixture is faint yellow). The volume of thiosulphate required (a) is noted. Simultaneously, the experiment is carried out without the oil or fat to get the blank value (b).

$$\text{Iodine value} = \frac{(b-a) \times 0.01269 \times 100}{\text{Wt in g of substance}}$$

If the normailty of the $Na_2S_2O_3$ solution is N, the above equation is multiplied by the factor $\frac{N}{0.1}$.

The approximate weight in g of the original substance needed may be calculated by dividing the figure 20 by the highest expected iodine value.

eg. expected iodine value of an oil is 80, then the amount to be taken for analysis $= \frac{20}{80} = 0.250$ g.

Reference :

British Pharmacopoeia Appendix X-B, p. 1955 (1963).

2. LIPIDS IN BIOLOGICAL TISSUES

a. Extraction of lipids

Tissues like liver are homogenised in a waring blender or Potter-Elvehjem homogeniser with at-least twenty volumes of a 2 : 1 mixture of chloroform-methanol. Aorta may be ground in a mortar with the chloroform-methanol mixture. Blood, serum or plasma is extracted with twentyfive volumes of chloroform-methanol mixture. From the tissue extract, the non-lipid matter is removed by Folch washing (0.2 volume of water to which is added different mineral salts). The lipid extract is made to a known volume with chloroform and required aliquots are used for the various determinations.

The stepwise procedure for the extraction of total lipids from blood plasma or serum is given below.

Extraction and purification of plasma total lipids: Into a 100 ml volumetric flask containing 32 ml methanol is pipetted 4.0 ml plasma through the sides with swirling of the flask. An equal volume (32 ml) of chloroform is then added and the mixture brought momentarily to boil by placing the flask for 1 min in a water bath at 70°C. More chloroform is then added, the flask is allowed to cool and the mixture is made upto the volume with chloroform. The contents are mixed well by inversion of the flask 20 times. The mixture is filtered into a 200 ml measuring cylinder through a chloroform-methanol washed filter paper (Whatman No. 41). The filter is washed with chloroform-methanol mixture and the washings are collected into the cylinder to make up the volume to 100 ml. Twenty ml of $CaCl_2$ solution (0.02%) or a saline solution (0.85%) are added to the mixture, the contents of the cylinder mixed by inversion 10 times and the stoppered cylinder is allowed to stand in cold (4°C) overnight to allow the layers to separate to clear solutions.

The upper aqueous methanol layer is then removed by suction with a water pump. If radioactive materials are likely to be present, then the lower chloroform layer is Folch washed 3 to 4 times with pure solvent upper phase (chloroform: methanol: water, 3 : 48 : 47) with 0.29% NaCl. After removing the upper phase 10ml of methanol is added and the cylinder inverted once to get a clear solution. The solution is then made upto a known volume with chloroform. Aliquots of this solution may be used for quantitative determination of total lipids, chemical estimation of cholesterol, triglycerides and phospholipids.

b. TLC of lipids

For separating the lipid classes, the whole lipid extract is evaporated under suction (or by blowing a stream of nitrogen) in a waterbath maintained at less then 50°C, to a small volume (5 to 10 ml). A rotary flask evaporator may also be used.

The concentrated lipid solution is transferred by means of a capillary pipette to a small storage tube (capacity about 10 ml) quantitatively with repeated washings with chloroform-methanol.

The solution is evaporated to dryness under N_2 at a temperature of 40°C and redissolved without exposing to atmospheric air in about 0.5 ml of chloroform.

If there is a necessity to store for long periods, the lipid can be stored under N_2 at –20°C.

(*i*) *Neutral lipids* : The TLC chamber is filled with the following solvents in the indicated proportions at least 1 hr prior to the placing of the TLC plates inside the chamber. Three of the sides of the chamber are covered with filter paper, the lower side of which dips into the solvent mixture.

The solvent system used is; petroleum ether: diethyl ether: acetic acid, 80 : 20 : 2.

The concentrated lipid extract is plated as adjacent spots to form a streak on one side of the heat activated TLC plate. Standard mixture of lipids (cholesterol ester, cholesterol, triglycerides, fatty acid and phospholipid) are spotted on either side of the sample spots. After allowing to dry in air for 3-5 min, the plate is kept inside the chamber and the solvent mixture allowed to ascend the plate to a distance of 2-3 cm from the top of the plate. The plate is taken out, allowed to dry in air for 5 min and a 0.2% solution of 2', 7'-dichlorofluorescein in alcohol is sprayed. After 5 min the separated bands are viewed under UV light and the fluorescing portions marked off with a pin. The order of separation of the lipid classes from the point of origin are as follows:

Origin; Phospholipids and monoglycerides; diglycerides; free cholesterol; free fatty acid; triglycerides and cholesterol ester; solvent front.

The bands corresponding to the respective lipid classes (identified from the position of the standards) are scraped off witn a sharp razor blade and collected carefully into a glass column through a funnel placed at the top. The following order of elution has been found to be satisfactory.

1. Cholesterol ester, triglycerides, free fatty acids and free cholesterol : 6 x 5 ml chloroform
2. Phospholipids: Eluted with the following solvent mixtures serially;
   (a) 3 x 5 ml of chloroform: methanol: acetic acid: water, 25 : 15 : 4 : 2
   (b) 4 x 5 ml methanol
   (c) 5 x 5 ml of methanol: acetic acid: water, 94 : 1 : 5

The eluates are concentrated in vacuo or under nitrogen at a temperature below 50°C and made upto a known volume with appropriate solvents. Suitable aliquots are taken for the chemical determination of the respective lipid classes or for preparation of methyl esters for studying the fatty acid composition by GLC.

(ii) Phospholipids: For the separation of phosphatidyl ethanolamine on TLC a basic silica gel plate is needed. For this 60 g of silica gel G and 120 ml of 0.01 M Na_2CO_3 solution are slurried and the plates are coated to a thickness of 500 μm. The plates are activated at 90°C for 1 hr before use.

Solvent system used is, chloroform: methanol: acetic acid: water, 50 : 25 : 8 : 4.

The TLC plate may be sprayed with a solution of 1% iodine in chloroform and the position of the separated spots may be marked. Left to stand for a while, the iodine will disappear and the marked portions may be scraped off, eluted or used directly for the preparation of fatty acid methyl esters for GLC.

c. Preparation of methyl esters of fatty acids of triglycerides for GLC

(i) With alkaline methanol: The triglyceride is usually extracted, in (1 : 2), methanol : chloroform washed with saline and made upto a known volume. After it is Folch washed, an aliquot is passed through a silicic acid column or through a column of silica gel G and eluted with chloroform. The eluate is concentrated and an aliquot containing about 10-25 mg of triglyceride is taken for the preparation of methyl esters of fatty acids.

The lipid extract is evaporated under N_2 at 50°C and 3 ml of 0.5 N NaOH (in dry methanol) is added. It is mixed well and kept at room temperature for 30-40 min at the end of which 0.3 ml of 6 N HCl is added, followed by 10 ml chloroform and 3 ml water. The methyl esters of the fatty acids are extracted into the chloroform layer by inversion of the tube 20-25 times. The upper aqueous methanol layer is discarded and the chloroform layer is washed twice with 5 ml water. The chloroform layer is then dried over anhydrous Na_2SO_4 and transferred into another tube for preservation under an atmosphere of nitrogen in the cold (-4°C or lower if possible).

The extract is concentrated or diluted with chloroform after an initial trial with 0.2% in GLC.

(ii) With methanolic HCl (5%): Hydrogen chloride gas is generated by letting concentrated H_2SO_4 kept in a separating funnel fall drop by drop on NaCl kept in a conical flask. The HCl gas is passed into a weighed amount of anhydrous methanol kept in a volumetric flask until there is the required increase in weight to yield a HCl concentration in methanol of 5% (W/W).

Silica gel portions from TLC plate corresponding to neutral lipid or phospholipid fractions are carefully scraped with a sharp razor blade and collected into a small glass vial with a teflon lined leak proof screw cap. Five ml of the 5% methanolic HCl solution is then added to the tube, the tube is closed airtight and kept in a hot air oven at 90°C for 1 hr. (The sample may also be taken in a glass ampoule, sealed well and then kept at 90°C for 1 hr). It is then cooled to room temperature, 3 ml water is added (followed by 10 ml petroleum ether, 40-60°C). The fatty acid methyl esters are extracted with the petroleum ether layer, the layer is washed free of acid with water, dried over anhydrous Na_2SO_4 and collected. It may be evaporated under nitrogen and redissolved in a suitable volume of chloroform for injection into the gas chromatogragph.

Reference :

J. Biol. Chem. **226**, 497 (1957).

3. TOTAL CHOLESTEROL

a. Liebermann-Burchard method

Principle :

The method involves:

(a) Treatment of serum with alcoholic KOH to liberate the cholesterol from lipoprotein complex and to saponify the cholesterol esters,

(b) extraction of cholesterol into a known volume of petroleum ether after dilution of the alcoholic solution with water and

(c) measurement of cholesterol in an aliquot of the petroleum ether extract by means of Liebermann - Burchard reagent.

Reagents :

1. 33% KOH (W/W): 10 g of KOH pellets dissolved in 20 ml water.
2. Alcoholic KOH: 6 ml of 33% KOH is made upto 100 ml with absolute alcohol. This solution is prepared fresh before use.
3. Standard cholesterol (40 mg/100 ml): 100 mg of cholesterol (recrystallised 4 times from ethanol and dried to constant weight) dissolved to make 250 ml with ethanol.
4. Modified Liebermann - Burchard reagent: 20 volumes of acetic anhydride is chilled to a temperature lower than 10°C in a glass stoppered container and 1 volume of concentrated H_2SO_4 is added. The well shaken mixture is kept for 9 min at the end of which is added 10 volumes of glacial acetic acid. The reagent is allowed to warm to room temperature and can be used within 1 hr.

Procedure :

Serum or plasma 0.5 ml is taken into a 25 ml glass stoppered test tube to which is added 5 ml freshly prepared alcoholic KOH. In the case of liver, the lipid residue from 2 g of the tissue is dissolved in 10 ml of petroleum ether and 1-2 ml aliquots are evaporated to dryness before adding alcoholic KOH. The tube is shaken well and then incubated in a water bath at 37-40°C for 55 min. After cooling to room temperature, 10 ml of petroleum ether is added and the stoppered tube inverted once to mix the contents. Five ml of water is then added and the tube is shaken vigorously for 1 min.

An aliquot is taken from the clear supernatant petroleum ether solution to contain 0.15-0.6 mg of cholesterol. The use of 0.5 ml of serum sample (for the cholesterol range of 150-300 mg/100 ml) permits 4 ml of the petroleum ether aliquot to be taken in duplicate. A 5 ml aliquot is taken from the liver extract.

The petroleum ether is evaporated by placing the tubes in a water bath at 60°C and blowing a gentle stream of dry air.

Standards are prepared for inclusion with each series of determination. This is most conveniently done by running the standard through the procedure along with the samples. Duplicate 5 ml aliquots of standard cholesterol solution (0.4 mg/ml) are taken and 0.3 ml of 33% KOH are added. The tube is then incubated for 55 min at 37-40°C. Ten ml of petroleum ether is added followed by 5 ml water and the tube vigorously shaken for 1 min.

Aliquots of 1, 2 and 3 ml of the petroleum ether extract are measured into test tubes and evaporated to dryness, to provide standards equivalent to 0.2, 0.4 and 0.6 mg of cholesterol respectively.

The tubes containing the dry cholesterol residue of sample and standard are arranged in such a way that one set of standard tubes appear at the beginning and another set at the end of the series. A clear empty tube is kept in the beginning as the blank. The samples are kept in a 25°C water bath. A stop watch is started and 6 ml of the Liebermann—Burchard reagent is added to the blank tube first and then at regular intervals of 1–1½ min to the other sample and standard tubes. The entire inner surface of the tubes is washed down with the reagent while pipetting and the tube is shaken and returned to the water bath maintained at 25°C in a dark chamber. The intensity of the colour in each tube is read at regular intervals against the blank in a photoelectric colorimeter at 620 nm. The readings should be taken at 28–32 min after the addition of the reagent.

Calculation :

The OD equivalent to 1 mg cholesterol is calculated from the standards.

$$\frac{\text{O D of standard}}{\text{mg of cholesterol in standard}} = S$$

Using the average of the values of different standard tubes the cholesterol in the unknown is calculated by the following relationship :

$$\text{mg of cholesterol/100 ml} = \frac{\text{OD of unknown}}{S} \times \frac{10}{\text{vol. of petroleum ether aliquot}} \times \frac{10}{\text{vol. of serum sample}}$$

Note :

The samples as well as the colour reagent should not be subjected to direct light during the colour development stage. However, usual laboratory lighting has little influence on the colour.

Reference :

J. Biol. Chem. **195**, 357 (1952).

b. $FeCl_3$ — H_2SO_4 method

Principle :

Cholesterol is oxidised by $FeCl_3$ — H_2SO_4. The purple colour thus formed is measured spectrophotometrically at 560 nm.

Reagents :

1. Standard cholesterol solution : 1 mg/ml in distilled ethanol.
2. $FeCl_3$ stock solution : 10 g of $FeCl_3$ in 100 ml acetic acid.
3. $FeCl_3$ — H_2SO_4 reagent : 2 ml of $FeCl_3$ solution diluted to 200 ml with conc. H_2SO_4.
4. 33% KOH (W/W) : 10 g of KOH dissolved in 20 ml water.
5. Alcoholic KOH solution : 6 ml of 33% KOH made upto 100 ml with distilled ethanol. This solution is prepared fresh before use.

Procedure:

Serum or plasma 0.2 ml is taken into 25 ml glass stoppered tube. Similarly 0.5 ml of standard cholesterol (500 μg) solution is taken in another tube. Add 5 ml freshly prepared alcoholic KOH solution. Shake the tubes well and incubate in a water bath at 37°C for 55 min. After cooling to room temperature, add 10 ml of petroleum ether and invert the tubes once to mix the contents. Then add 5 ml glass distilled water and shake the tubes vigorously for 1 min. Take 0.5 — 2.0 ml aliquots from the supernatant (petroleum ether) into test tubes. Evaporate the petroleum ether extract under nitrogen,

To each of the sample as well as standard tubes including a blank, add 3 ml of glacial acetic acid followed by 0.1 ml glass distilled water. Mix the tubes thoroughly. Add 2 ml of the $FeCl_3 — H_2SO_4$ reagent to the sides of the tubes, a brown ring is formed at the interface. Tap the bottom of the tubes well, to effect mixing. A light colour appears which changes to an immense purple colour which is measured in a spectrophotometer at 560 nm.

Calculations:

mg cholesterol/100 ml serum =

$$\text{O D of sample} \times \frac{100}{\text{OD of standard for 100}\mu\text{g}} \times \frac{\text{total volume of petroleum ether extract}}{\text{aliquot of petroleum ether extract taken}} \times \frac{100}{\text{quantity of serum in ml}} \times \frac{1}{100}$$

Reference:

J. Lab. Clin. Med. **41**, 486 (1953).

4. POLYUNSATURATED FATTY ACIDS

Principle:

The procedure involves the participation of the hydrogen atoms at the methylene group between the double bonds that are more reactive than the rest of the hydrogen atoms in the fatty acid chain. The labile hydrogen atoms cause a shift of double bonds resulting in the formation of a conjugated double bond system, which has a characteristic absorption maxima in the U V region.

Reagents:

1. Ethylene glycol (E. Merck): Distilled over NaOH before use.
2. Bloor's mixture, ethanol-ether (3 : 1): Peroxide free ether and freshly distilled alcohol (95%) are mixed each day in the ratio of 1 : 3.
3. 21% KOH in ethylene glycol: 100 ml of freshly distilled ethylene glycol is heated to 190°C for about 10 min and allowed to cool to 150°C. 28 g of 85% KOH is added to the ethylene glycol and the solution is reheated to 190°C for about 10 min and then cooled to room temperature. All these manipulations are done under an atmosphere of nitrogen. The strength of the solution is determined titrimetrically and adjusted, if necessary to 21.0 $\pm$ 0.1%. The reagent can be stored under nigtrogen at—5°C in glass stoppered bottles for many months.
4. Collection and preservation of the sample: Venous blood is drawn and collected in heparinised tubes under basal conditions. It is centrifuged to separate the plasma. The erythro-

cytes are washed thrice with physiological saline and haemolysed by the addition of equal quantity of distilled water and left overnight at 0°C.

The animals are bled after an overnight fast by cardiac puncture under light anesthesia. Tissues are removed, washed free of blood with normal saline, and dried by pressing between folds of filter paper. The tissue is then stored in a polythene bottle containing 5 10 ml of absolute alcohol to which 0.05 mg of hydroquinone is added. After passing nitrogen for 2 – 4 min the bottle is stoppered tightly and kept at – 5°C until anaylysis.

Procedure :

Extraction of the material : To 15–20 volumes of Bloor's mixture 1 volume of plasma or 2 volumes of haemolysate are added dropwise with constant shaking.

After adding 0.01 mg hydroquinone, the contents of the flask are allowed to boil for 1 – 2 min by placing the flask in a beaker containing boiling water. After cooling to the room temperature, the contents are made upto 25 volumes with Bloor's mixture and filtered through a fat free Whatman No. 1 filter paper into a bottle and stored at - 5°C for not more than a week.

The tissue is macerated with anhydrous Na_2SO_4 or with glass powder in a mortar along with 10-15 volumes of Bloor's mixture and transferred into a glass stoppered measuring cylinder and left overnight. It is then made upto the mark and after shaking vigorously, filtered into a polythene bottle through Whatman No. 1 filter paper (fat free). The extract can then be stored at –5°C for not more than a week.

Purification of lipid extract : One volume of Bloor's extract and half its volume of 0.5% NaCl solution are pipetted into a 250 ml seperating funnel. The mixture is extracted once with one volume of petroleum ether and twice with half volumes of petroleum ether. A few drops of alcohol may be added to break the emulsions if any. The combined petroleum ether extracts are washed thrice with equal volumes of distilled water, using NaCl solution to break the emulsions. The extract is dried with anhydrous sodium sulphate overnight, filtered and concentrated to about 2 ml under suction.

The lipids in petroleum ether are transferred to a 10 ml volumetric flask using several rinses of petroleum ether and the volume made upto the mark. The optical density of this solution is measured at 375, 346, 315, 268 and 233 nm in a Beckman D U spectrophotometer against a solvent blank.

Isomerisation procedure : Two ml or a suitable aliquot of the solution is transferred to a pyrex tube (reaction tube) and the solvent is evaporeted under suction, 1 ml of absolute ethanol, 0.64 ml of KOH-glycol reagent are added and the mouth of the tube is immediately covered with a glass marble and the contents are throughly mixed. The air in the tube is replaced with nitrogen gas. The tubes are then placed in an 180°C copper block. Exactly 20 min later the tube is removed from the bath and placed directly in ice water. A tube with reagent and ethanol only, which serves as a blank is run with each set of samples.

The isomerised samples and the blank are diluted gradually with methanol and mixed by vigorous shaking at each step. The volume is then made upto 5 ml. Optical density measurements are made with this solution or an appropriate diluted solution of the same, at 375, 346, 315, 268 and 233 nm using the corresponding diluted blank as reference. The slit widths employed at the different wavelengths are 0.22, 0.22, 0.3, 0.5 and 1.0 respectively.

Calculations :

In calculating the content of polyunsaturated fatty acids/100 ml blood, plasma etc, and per 100 g tissue, the following equations are used,

mg decosahexaenoic acid (22:6) = 4.186 OD_{375} - 0.1778 OD_{346}

mg eicosapentaenoic acid (20:5) = 1.559 OD_{346} - 1.628 OD_{375}

mg arachidonic acid (20:4) = 1.450 OD_{315} - 1.314 OD_{346} - 0.4128 OD_{375}

mg linolenic acid (18:3) = 1.266 OD_{268} - 0.8028 OD_{315} + 0.3172 OD_{346} - 1.778 OD_{375}

mg linoleic acid = 1.087 OD_{233} - 0.615 OD_{268} - 0.1354 OD_{315} - 0.1072 OD_{346} - 0.412 OD_{375}

References :

1. Nutrition Research Laboratories. Annual Report, p. 40 and 86 (1966).
2. Methods of Biochemical Analysis. David Glick (Ed.) Interscience publications Ltd., London Vol. 4, p. 126 (1957).

5. GLYCOLIPIDS

Principle :

Glycolipids are estimated by determining the hexose part and multiplying it with 4.45.

Reagent :

1. 2% and 80% phenol solutions.
2. Standard galactose 1 mg/ml water.

Procedure :

Different concentrations (20-200 μg) of galactose standard solution are taken in test tube and 1 ml of 2% phenol is added, followed by 4 ml of conc. H_2SO_4. Orange colour appears, which is measured, after cooling to room temperature for 15 min at 480 nm.

For biological samples, hydrolysis is done with 2 ml 2 N H_2SO_4 for 2 hr. (The time can be varied depending upon the source of sample). After hydrolysis is over, 4 ml of chloroform is added and the mixture centrifuged. From the top aqueous layer 1 ml is taken out separately. To this 50 μl of 80% phenol is added followed by 4 ml of conc. H_2SO_4. The orange colour developed is measured at 480 nm and the concentration of the galactose part of the lipid is calculated from the standard curve. This is then multiplied by 4.45 to estimate the glycolipids.

Reference :

Anal. Biochem. 22, 74 (1968).

6. TRIGLYCERIDES

Principle :

Glycerol moiety is oxidised to formaldehyde and the latter condensed with ammonia and 2, 4–pentanedione (acetyl acetone) to produce 3, 5–diacetyl 1, 4– dihydrotoludine which is yellow in colour and has absorption at 405 nm.

Reagents :

1. Alumina (neutral-chromatography grade) : Wash the alumina with water until all the fine particles are removed. Dry in an oven at 100 — 110°C overnight, cool and store in a desiccator. Stable for six months.

2. Saponification reagent: Dissolve 5.0 g KOH in 60 ml distilled water and add 40 ml isopropanol.
3. Sodium metaperiodate reagent: To 77 g of anhydrous ammonium acetate in 700 ml distilled water, add 60 ml glacial acetic acid and 650 mg of sodium metaperiodate. Dissolve and dilute to 1 l with distilled water.
4. Acetyl acetone reagent: Add 0.75 ml of acetyl acetone to 20 ml of isopropanol and mix well. Add 80 ml of distilled water and mix.
5. Stock standard solution: 4 mg/ml of triolein.

Procedure:

Extraction: Take 0.1 ml of serum or plasma and standards in screw capped tubes. Make up the volume to 4.0 ml with isopropanol. Mix well and add 0.4 g of washed alumina (a calibrated scoop may be used) to all the tubes. Place them on mechanical shaker for 15 min and centrifuge.

Transfer 2.0 ml of supernatant fluid after centrifugation to appropriately marked tubes. Add 0.6 ml of saponification reagent to the tubes and incubate at 60 - 70°C for 15 min. After cooling add 1.0 ml of sodium metaperiodate solution and mix well. Then add 0.5 ml acetyl acetone reagent and mix again. Incubate the tubes at 50°C for 30 min. After cooiing read the colour at 405 nm in a spectrophotometer.

Calculation:

$$\text{mg triglyceride/100 ml serum} = \text{O D of sample} \times \frac{200}{\text{O D of standard}} \times \frac{4}{2} \times \frac{1}{1000} \times \frac{100}{0.1}$$

References:

1. J. Lab. Clin. Med. **50**, 152 (1957).
2. Clin. Chem. **19**, 338 (1973).

7. CEREBROSIDES

Principle:

Cerebrosides are separated from total lipids by TLC and quantitated by determining the hexose content after hydrolysis with concentrated sulphuric acid. A factor of 4.55 is used to convert hexose value to cerebroside.

Reagents:

1. 3N H_2SO_4
2. 50%. Distilled phenol

Procedure:

Separation from total lipids: Pure cerebrosides are separated by TLC from total lipids using a solvent system comprising of chloroform-methanol (100 : 20). This system ensures complete separation of cerebrosides from other lipid constituents. Lipid constituents are detected by exposure to iodine vapours. Standard bovine cerebrosides are run simultaneously under these conditions. Distinct double spots characteristic of cerebrosides with Rf value of 0.55 will be evident. Areas corresponding to

cerebrosides are scraped off after sublimation of iodine. Four 5 ml portions of chloroform-methanol, 2 : 1 followed by two 5 ml portions of acetone are used to ensure complete elution from silica gel G.

Hydrolysis : Eluted samples of cerebrosides are evaporated to dryness. To the residue in a centrifuge tube 2 ml of 3N H_2SO_4 is added and kept in boiling water bath for 4 hr, cooled and added 4 ml of chloroform and centrifuged. To 1 ml of supernatant 0.04 ml of 50% phenol and 4 ml of conc. H_2SO_4 are added and mixed thoroughly. The colour developed is read in a spectrophotometer at 480 nm. Analar galactose in the range of 20 – 100 μg treated with phenol and conc. H_2SO_4 serves as standards. A factor of 4.55 is then used to convert galactose values to cerebrosides.

References :

1. J. Neurochem. **1**. 42 (1956).
2. Anal. Biochem. **22**, 74 (1968).

8. FREE FATTY ACIDS

Principle :

Free fatty acids are extracted from lipids by using Dolls extraction mixture (40 : 10 : 1, heptane : isopropanol : acetic acid). Then the heptane phase is taken and evaporated. The free fatty acids form a complex with cupric ions when mixed with copper reagent, the coloured complex formed with copper is soluble in chloroform and diethyldithiocarbamate is used as a colour developer.

Reagents :

1. Copper reagent : This consists of 9 volumes of aqueous 1 M triethanolamine, 1 volume of 1N acetic acid and 10 volumes of 6.45% $Cu(NO_3)_2 .3H_2O$.
2. Sodium diethyl dithiocarbamate : The diethyldithiocarbamate reagent is 0.1% (W/V) solution of sodium diethyldithiocarbamate in redistilled secondary butanol.
   (Both the reagents are stored in the refrigerator and use within seven days).
3. Standard fatty acids solution : Make up in chloroform in the range 10-100 μM myristic acid, palmitic acid, stearic acid and linoleic acid.

Procedure :

Take about 5 ml of the chloroform solution of fatty acids and place in centrifuge tubes. Add 2.5 ml of copper reagent. The tubes are stoppered and shaken vigorously for 1 min. Centrifuge the tubes for a few min. Remove the supernatant aqueous phase by suction with a fine hypodermic needle. The surface of the chloroform phase can easily be left clean with only traces of aqueous phase adhering to the wall of the tube. A portion (2.5 ml) of chloroform layer is taken into a clean dry tube, care should be taken that the pipette do not touch the inner wall of either tube, as traces of copper containing aqueous phase might be transferred. Then 0.5 ml of diethyldithiocarbamate reagent is added to the chloroform solution and mixed. The extinction is read at 440 nm. All measurements are made against a blank solution that had been subjected to the same procedure.

Reference :

Biochem. J. **88**, 7 (1963).

9. PHOSPHOLIPIDS

Principle :

The organic phospholipid phosphorous is converted to inorganic phosphorous which reacts with

ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ANSA forms a stable blue colour.

Reagents :

1. 2.5% Ammonium molybdate : Dissolve 25 g of reagent grade ammonium molybdate in about 200 ml of water. Transfer it to a 1 l volumetric flask containing 300 ml of 10N H_2SO_4 and make up the volume with water (stable indefinitely).
2. Fiske-Subba Row reagent : To 195 ml of 15% sodium bisulfite solution add 0.5 g of 1, 2, 4-aminonaphthosulfonic acid. Add 5 ml of 20% sodium sulfite and mix well. Filter this solution and store in an amber coloured bottle (stable for 4 weeks).
3. Standard phosphorous solution : Dissolve 0.351 g of monopotassium phosphate in 10 ml of 10N H_2SO_4 and make up the volume to 1 l with distilled water. This solution contains 0.4 mg of Pi/5 ml and is stable indefinitely.

Procedure :

Evaporate lipid fractions to dryness and add 0.5 ml of 10N H_2SO_4. The samples are placed in an 150-160°C oven for at least 3 hr. Two drops of fuming HNO_3 are added and the solution is returned to the oven for at least $1\frac{1}{2}$ hr more to complete the combustion. The samples are removed and cooled. Ammonium molybdate solution 4.6 ml is added followed by 0.2 ml of Fiske-Subba Row reagent. The tubes are covered with marbles and heated for 7 min in boiling water bath. The OD at 660 nm is recorded with the use of a Beckman model DB spectrophotometer or in Junior Coleman spectrophotometer.

Note :

The colour produced is proportional to the concentration of phosphorous upto 1.5 μ moles in the reaction mixture. These values may be expressed as the phospholipid (Lecithin) by multiplying by a factor of 25.

Reference :

J. Biol. Chem. **234**, 466 (1959).

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V. NUCLEIC ACIDS AND NUCLEOTIDES

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1. NUCLEIC ACIDS

a. Isolation of nucleic acids

Principle :

The procedure involves the isolation of nucleic acids with hot TCA from tissues. The extract contains both DNA and RNA.

Reagents :

1. TCA : 5% and 10% in glass distilled water.
2. NaCl : 0.9% in glass distilled water.

Procedure :

Tissues : A 5% homogenate of the tissue is made in 0.9% saline. This is filtered through four layers of cheese cloth and use the filtrate for further isolation.

Blood : A known volume of fresh blood is centrifuged at 1000 rpm for 5-10 min and the pellet containing RBC is washed several times (3-4 times) with 0.9% NaCl and finally suspended in the same solution.

A known volume of the extract (200 mg wet tissue/ml) made as above is taken and treated with equal volume of 10% TCA. The solution is shaken well centrifuged and the supernatant discarded. The residue is washed twice with 10% TCA followed by washing with 5 ml of absolute enthanol twice. After this, the residue is ether extracted twice to remove the phospholipids.

The following acid extraction is done next.

a) Add 2.5 ml of 10% TCA, mix and centrifuge and save the supernatant in a separate test tube.

b) The residue from (a) is treated with 5 ml of 5% TCA, keep at 100°C for 15-20 min, cool, centrifuge and add the 5 ml supernatant to (a).

c) The final residue is treated with 2.5 ml of 5% TCA, heat it as above, and save the final supernatant.

From the total 10 ml of the clear acid extract, aliquots are used for DNA and RNA determination.

b. Estimation of DNA

Principle :

The deoxyribose moiety of DNA forms w-hydroxy laevulaldehyde in TCA solution. This reacts with diphenylamine to give a blue colour which can be read at 565 nm.

Reagents :

1. Dische's reagent : Dissolve 1.5 g of crystalline diphenylamine in 100 ml of glacial acetic acid and add 1.5 ml of conc. H_2SO_4. The reagent is stored in the dark. At the time of use add 0.1 ml of aqueous acetaldehyde (16 mg/ml) for each 20 ml of the reagent.

2. 1N Perchloric acid

3. 5 mM NaOH

4. Standard DNA solution : A stock solution is prepared by dissolving DNA (0.4 mg/ml) in 5mM NaOH. Working standards are prepared every three weeks by mixing a measured volume of the stock standard with an equal volume of 1N perchloric acid and heating at 70°C for 15 min.

Procedure :

A standard curve is made by pipetting different aliquots of standard DNA solution, containing 50, 100, 200 and 500 μg of DNA into clean tubes. Separately, 0.1 – 1.0 ml of the acid extract is taken in clean tubes. To this, 2.0 ml of the freshly made Dische's reagent is added and the total volume in each tube is made upto 5.0 ml with glass distilled water. The initial turbidity formed disappears gradually. The tubes are covered with glass marbles and heated in a boiling water bath for 10-15 min. The solution is then cooled under tap water and the blue colour developed is measured at 565 nm in a colorimeter or Beckman spectrophotometer.

The control (or balnk) contains only water and the Dische's reagent.

Calculation :

From the standard curve the value of DNA is computed for a definite volume of the extract used and finally calculated for the initial 10 ml acid extract made. This being the amount present in 1.0 ml of the tissue extract (200 mg wet weight of the tissue, or the RBC content of the volume of blood taken initially or the total protein of the same). Final values may be expressed as percentages of the tissue weight or the organ weight.

c. Estimation of RNA

Principle :

The ribose moiety of RNA in the TCA extract is converted to furfural which condenses with orcinol to yield a green colour that can be measured at 675 nm.

Reagents :

1. 0.1% $FeCl_3$ solution in concentrated HCl.
2. 0.2% Orcinol solution in absolute alcohol.
3. 0.1 M NaCl

4. Standard RNA solution : Dissolve yeast crystalline RNA in 0.1 M NaCl (5 mg/ml). If cloudy, add few drops of 0.1N NaOH, filter and store in cold.

Procedure :

The method adopted here is the same as for DNA in terms of the aliquots of the acid extract used for the analysis. Take standard solution containing 100, 200, 400 and 800 μg of RNA in different aliquots in clean tubes. To these tubes 3.0 ml of the ferricchloride reagent is added and the total volume is brought to 5.5 ml with glass distilled water prior to the addition of orcinol reagent. Freshly prepared orcinol reagent in ethanol, 0.3 ml is added and all the tubes are kept in a boiling water bath for 20 min. The green colour developed, if clear is read at 675 nm against a blank treated in the same manner. If turbid, it is extracted with 5 ml of isoamyl alcohol and the colour read.

Calculation :

The value obtained is compared with that from a standard RNA solution. As in the case of DNA, final values are calculated for 1 g of wet tissue or as percentage.

Note :

In this method, interference by DNA is only 0.85% as compared to 12% by other procedures found in the literature.

References :

1. J. Biol. Chem. **161**, 293 (1945).
2. Methods of Biochemical Analysis, David Glick (Ed.) Vol. VI, p. 10 (1958).

2. ESTIMATION OF ADENOSINE TRIPHOSPHATE

Principle :

Aqueous extracts of firefly lanterns which are no longer luminous emit light on the addition of ATP. The other requirements for the reaction are a divalent cation Mg^{++} and a fluorescent compound luciferin. Luciferase preparations are available commercially.

$$\text{ATP} + \text{Luciferin} \xrightarrow[Mg^{++}]{\text{Luciferase}} \text{Adenyl} - \text{luciferin} + \text{Pyrophosphate}$$

$$\text{Adenyl-luciferin} \xrightarrow{O_2} \text{Adenyl} - \text{oxyluciferin} + H_2O + \text{light.}$$

The light emitted is directly proportional to ATP concentration.

Reagents :

1. 0.16 M ATP : Dissolve 25 mg of ATP (disodium salt) in 250 ml water. Store at – 20°C. Preferably prepare fresh standards. Dilute 1 in 100 before use.

2. 0.1 M Arsenate – 50 mM Mg buffer, pH 7.4 : Dissolve 42.5 g $Na_3AsO_4 . 12H_2O$ and 10 g $MgCl_2 . 6H_2O$ in 500 ml glass distilled water. Adjust the pH to 7.4 with 1N HCl and dilute to 1 l.

3. Luciferase : Grind 50 mg of vacuum dried firefly lanterns (Sigma Chemical Co. 10 or 12 lanterns) with 5 ml of ice cold 0.1M arsenate buffer for 5–10 min. Filter the mixture into a test tube standing in ice bath. Dissolve 50 mg $MgSO_4.7H_2O$ in the filtrate. The preparation is stable for several days at 4°C.

4. Phenazine methosulfate (PMS) 0.5 mM : Required only if rate of ATP production is to be followed. Dissolve 0.145 g of PMS in 1 l water.

Procedure :

Preparation of the sample from tissue or mitochondria : Heat the sample at 100°C in boiling water bath for 5–10 min. To avoid the enzymatic degradation of substrates in the sample during the heat inactivation bring the samples to 100°C quickly. This may be achieved by adding quickly the sample into 2 or 3 volumes of boiling water. Immediately after heating transfer the same to 0°C.

Take 0.2 ml luciferase solution and 0.6 ml water. Immediately before the measurement, add 0.2 ml of sample, mix rapidly and read the light intensity upto 30 sec.

Measurements can be made on Farrand fluorimeter or scintillation counter.

Calculation :

The luminiscence obtained is directly proportional to ATP concentration in the physiological range, he light intensity produced by the sample is compared to that produced by the standard. Construct a standard curve of your own to establish the linear relationship between luminiscence and ATP concentration.

References :

1. Methods of Enzymatic Analysis, B. L. Strehler (Ed.), Vol. IV, p. 2112 (1974).
2. Anal. Biochem. 68, 187 (1975).

. ESTIMATION OF CYCLIC ADENOSINE MONOPHOSPHATE

Principle :

Cyclic adenosine monophosphate (c–AMP) binds noncovalently with the regulatory subunit of -AMP dependent protein kinases from various mammalian tissues. This property is made use of in the mpetitive protein binding assay for c-AMP.

Reagents :

1. 20 mM Tris-HCl buffer, pH 7.5 : Dissolve 2.422 g of Tris in about 600 ml of deionised water, just the pH to 7.5 with HCl and make up the volume to 1 l.

2. 50 mM sodium acetate buffer, pH 4.5 : Prepare 50 mM acetic acid (2.89 ml of glacial acetic d in 1 l water) and 50 mM sodium acetate (4.1 g of sodium acetate or 6.89 g of sodium acetate trihyte in 1 l water). Add sodium acetate solution to acetic acid till pH 4.5 is obtained.

3. 500 mM sodium acetate buffer containing 8 mM EDTA, pH 4.0: Prepare 500mM acetic acid 8.9 ml of glacial acetic acid in 1 l water) and 500 mM sodium acetate (41 g of sodium acetate or 68.9 g

of trihydrate in 1 l water). Add sodium acetate solution to acetic acid till pH 4.0 is obtained. In 1 l of this buffer dissolve 2.978 g of disodium EDTA.

4. 5 % TCA.

5. c-AMP Standard solution: Prepare a stock standard solution by dissolving 3.292 mg of c-AMP in 100 ml water (100 μM). Dilute this solution to 100 times before use. This contains pmole of c-AMP/μl.

6. [^{3}H] c-AMP: Dilute the commercially obtained [^{3}H] c-AMP (sp. activity, 16-28 Ci/ mmole) with water such that 20 μl of it gives 10, 000 to 20, 000 cpm.

7. 20 mM phosphate buffer containing 10 mM $MgCl_2$, pH 6.0: Prepare 20 mM K_2HPO_4 (3.484 g/l) and 20 mM KH_2PO_4 (2.722 g/l). Adjust the pH of KH_2PO_4 solution to 6.0 using K_2HPO_4 solution. In 1 l of this buffer dissolve 2.033 g $MgCl_2.6H_2O$.

Procedure :

Sample preparation : Urine samples generally contain high amounts of c-AMP and can be used as such in the assays or after suitable dilution with water. Tissues are homogenised in 5% TCA and the precipitate is removed by centrifugation. The supernatant is extracted with diethyl ether (6 times with 2 to 3 volumes) to remove TCA and the residual ether is removed by heating at 80-90°C for 2-5 min. The samples are lyophilised and redissolved in 50 mM sodium acetate buffer, pH 4.5 prior to assay.

Preparation of binding protein : Bovine kidney is rich in binding protein and can be homogenised easily. The partially purified binder preparation is stable for several weeks at –20°C. Bovine kidneys (fresh or stored at -20°C) are homogenised in 3 volumes of 20 mM Tris-HCl (pH 7.5) and the homogenate centrifuged at 40,000 g for 30 min. The supernatant is subjected to ammonium sulphate fractionation and the fraction precipitating between 30-45% saturation is dialysed extensively against Tris-HCl (20 mM, pH 7.5) and stored at –20°C until use (The amount of solid $(NH_4)_2 SO_4$ to be used is equal to 0.707g/ per 100 ml of starting solution).

In 5 ml test tubes take 20 μl of 500 mM sodium acetate buffer pH 4.0, 20 μl of [^{3}H] c-AMP, standards (2.5–20 p moles) and appropriately diluted aliquots of unknowns. A total volume of about 80 μl is reserved for these components in the incubation mixture, ie. for standards and unknowns. Necessary amounts of water are added to all tubes such that a constant volume of incubation mixture is obtained. The tubes are placed in an ice bath and the binding protein is added to a total volume of 200 μl, the contents are mixed gently and allowed to incubate for atleast 1 hr at 0°C. The amount of binding protein should be sufficient to bind 60-80% of total counts of [^{3}H] c-AMP such that sufficient amount of [^{3}H] c-AMP is left to ensure saturation of the binding protein. The reaction mixture is then diluted with 4 ml of 20 mM potassium phosphate buffer, pH 6.0 at 0-3°C and are passed under gentle suction through 25 mm cellulose ester millipore filter (pore size 0.45 μm) previously rinsed with the same buffer. The filter is washed 2-4 times with 4 ml aliquots of phosphate buffer to remove unbound c-AMP and placed in counting vials. Bray's mixture dissolves the filters and the vials are counted after keeping overnight in darkness.

Reference :

Biochem. Biophys. Res. Comm. 46, 99 (1972).

VI. VITAMINS COENZYMES AND METABOLITES

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1. MICROBIOLOGICAL ASSAY

a. Preparation of common solutions

1. Acid hydrolysed casein : To 100 g of casein is added enough 95% alcohol and the mixture refluxed for 2–3 hr and filtered. This process is repeated twice more. After removing the alcohol at room

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temperature, 250 ml concentrated HCl and 250 ml distilled water are added to the casein which is then refluxed for 8—10 hr. The HCl is distilled off under vacuum at a temperature not exceeding 80°C. The residue is redissolved in distilled water and the water removed by evaporation in vacuum as above. This process is repeated 2-3 times for complete removal of HCl. The hydrolysed casein is dissolved in about 600 ml water and the pH adjusted to 3.5 with 40% NaOH and filtered. The filtrate is stirred with 20 g of activated charcoal, for 15 – 30 min at room temperature, filtered and stored in the refrigerator under toluene.

2. L – Cystine : 4.0 g of L – cystine is made upto 500 ml with water, using concentrated HCl to dissolve the amino acid.

3. DL – Tryptophan : 2.0 g of DL - tryptophan is dissolved in water using concentrated NH_3 to aid solution. The pH is adjusted to 3.5 and the volume made upto 500 ml. It is then stirred with activated charcoal (2 g/100 ml) for 10-15 min, filtered and stored in the cold under toluene.

4. Pyridoxine and Thiamine : 50 mg of pyridoxine and 20 mg of thiamine are dissolved in water to make upto 100 ml in separate containers.

5. Biotin : 25 mg of biotin is taken up in 500 ml of 50% alcohol in water.

6. Riboflavin : 20.0 mg of riboflavin is made upto 100 ml, using acetic acid to help solution. If necessary the solution may have to be heated on a water bath.

7. Nicotinic acid : 50 mg in 500 ml of 50% alcohol in water.

8. p – Aminobenzoic acid (PABA) : 12.5 mg in 100 ml water.

9. Folic acid : 10 mg in 100 ml 0.8% sodium bicarbonate solution.

10. Calcium pantothenate : 54.4 mg in 500 ml water.

11. Vitamin solution :

Riboflavin	—	20 mg
Thiamine HCl	—	10 mg
PABA	—	10 mg
Pyridoxine HCl	—	40 mg

Dissolved in 1 l of 0.02 N HCl.

12. Peptone solution : 10 g of Difco peptone is dissolved in 80 ml water, the pH adjusted to 3.0 with HCl and the volume made upto 100 ml. The solution is stirred with 5 g of activated charcoal for 1 hr and filtered. This process is repeated twice more with 2 g of charcoal.

13. DL – Alanine : 2 g in 100 ml water.

14. Salt A, salt B, AGU and xanthine solutions are as described under amino acids-microbiological assay.

b. Riboflavin

Microorganism : *Lactobacillus Casei* (A. T. C. C. No. 7469).

Stock riboflavin standard : 25.0 mg of riboflavin is dissolved in water, 1.2 ml of glacial acetic acid is added and the volume made upto about 800 ml with distilled water. The solution is heated to dissolve the crystals, cooled and made upto 1 l with water (25 μg riboflavin/ml).

Working standard : For riboflavin assays the standard range is 0 – 0.2 μg riboflavin. The stock riboflavin standard solution is diluted to get a working standard containing 0.04 μg/ml.

Reagents :

Solutions 1 to 4, 7 to 10, 12 to 14 are prepared as mentioned earlier under 'Preparation of common solutions'.

Basal medium : The following solutions are mixed.

		in 100 ml
Casein hydrolysate	—	10 ml
Peptone	—	0.4 ml
DL – Tryptophan	—	10 ml
L – Cystine	—	5 ml
DL – Alanine	—	1 ml
Salt A	—	5 ml
Salt B	—	1 ml
AGU Solution	—	1 ml
Xanthine	—	1 ml
Biotin (Stock solution 1.5 ml diluted to 100 ml)	—	1 ml
Thiamine, pyridoxine	—	1 ml each
Nicotinic acid	—	2 ml
Calcium pantothenate	—	2 ml
PABA	—	0.3 ml
Folic acid	—	1 ml
Glucose	—	4 g
Sodium acetate $3H_2O$	—	6.64 g

The pH is adjusted to 6.8 before making up the volume to 100 ml. The solution is then filtered.

Preparation of samples :

Foodstuffs: Into a 150 ml conical flask a homogeneous sample containing 10 μg or more of riboflavin is weighed. Fifty ml of 0.1 N HCl is added, the mixture autoclaved for 15 min at 15 lb pressure, cooled to room temperature and transferred to a beaker. The pH is adjusted to 4.5 and the solution made upto a known volume. It is then mixed well and filtered. (For most samples filtration at pH 4.5 is effective in removing growth stimulants and inhibitors such as starch and fatty acids). In the case of materials containing high amounts of fat, the extracts after adjusting to pH 4.5, may be shaken with distilled ether (the extraction with ether may be repeated three or four times). A known volume is taken, the pH adjusted to 6.8 and diluted to get a final concentration of around 0.04 μg riboflavin/ml. For assays 1, 2, 3 and 4 ml levels are taken in duplicate and 5.0 ml basal medium is added to each assay tube.

Urine: Urine is collected in dark bottles containing HCl and toluene. A known volume is taken in a beaker, the pH adjusted to 6.8 and the sample diluted to contain around 40 ng riboflavin/ml. One to 4 ml aliquots are taken for the assay and each diluted to 5 ml. To each tube 5 ml of basal medium is added. The tubes are autoclaved at 12 lb for 12 min, cooled, inoculated with washed cells of *L. Casei* and incubated for 36 hr. Growth is measured turbidimetrically.

Red blood cells : Red blood cells are separated from heparinised blood, washed with 0.9% saline, diluted with an equal volume of water and frozen at – 20°C overnight.

One ml of the haemolysate is diluted with 4 ml water and 1, 2, 3 and 4 ml aliquots taken for the assay. Water is added to bring the volume to 5 ml and 5 ml of basal medium is added.

After inoculation with the organism, the tubes are incubated for 72 hr. The samples are filtered and the filtrate collected. Each assay tube is washed twice with distilled water. To the combined filtrate and washings 6 drops of 0.1% bromothymol blue (BTB) are added, and the acid produced titrated against 0.1 N NaOH to pH 6.8.

Liver : Weighed portion of the liver is hydrolysed with 25 volumes of 0.1 N HCl by autoclaving for 15 min at 15 lb. The pH of the hydrolysates is adjusted to 4.5 and samples stored at 0°C. Suitable aliquots of this hydrolysate are used for the assay after adjusting the pH to 6.8.

Tubes are incubated for 36 hr after inoculation and read turbidimetrically.

Standard levels : The working riboflavin standard 0, 0.5, 1, 2, 3, 4 and 5 ml is used in duplicate. Incubation period can be 36 hr for turbidimetric determination, the tubes being read at 660 nm

References :

1. Biochem. J., 50, 326 (1952).
2. Methods of Vitamin Assay. The Association of Official Vitamin Chemists (Ed.), 3rd Edn. p. 147 (1966).

c. Niacin

Microorganism : *Lactobacillus Arabinosus* (A. T. C. C. No. 8014).

Reagents :

Niacin stock standard solution : 50 mg of niacin is diluted to 500 ml with 50% alcohol to give 100 μg/ml.

Working Standard (0 – 0.5 μg/ml) : The stock standard solution is diluted to give a final concentration of 0.1 μg/ml.

Solutions No. 1, 2, 3, 5, 10, 11 and 14 (AGU, Salt A and Salt B solutions) are prepared as described earlier.

Basal medium		
Casein hydrolysate	—	10 ml
DL - Tryptophan	—	5 ml
L-Cystine	—	5 ml
AGU Solution	—	1 ml
Vitamin solution	—	2 ml
Salt A	—	1 ml
Salt B	—	1 ml
Calcium pantothenate	—	0.2 ml
Biotin (1 : 500 of 50 μg/ml water)	—	0.8 ml
Glucose (Anhydrous)	—	2 g
Sodium acetate. 3 H_2O	—	3.32 g

The above ingredients are dissolved in water, the pH adjusted to 6.8 and the volume made upto 100 ml. The solution is then filtered.

Preparation of samples :

Food stuffs : Sufficient material is weighed (to contain approximately 0.1 mg of niacin) into a conical flask to which is added 50-100 ml of 1 N H_2SO_4. The mixture is autoclaved for 30 min at 15 lb pressure, cooled, the pH adjusted to 4.5, made upto a known volume and filtered. An aliquot is taken, the pH adjusted to 6.8 and diluted to get a final concentration of about 0.1 μg niacin/ml. Volumes 0.5, 1,2 and 3 ml are used in duplicate for the assays. Standard levels 0, 0.5, 1, 2, 3, 4 and 5 ml (in duplicate) of the working standard niacin are used. Incubation period lasts 18–20 hr for turbidimetric determination.

Urine : A 24 hr urine collection is made over 5 ml of glacial acetic acid. An aliquot of the urine can be preserved if necessary under toluene in the cold.

Five ml of the filtered urine is taken in a beaker and the pH is adjusted to 6.8. The volume is made upto 50 ml. In duplicate tubes, 0.5, 1.0 and 2.0 ml of the diluted urine samples are taken. Sufficient water is added to bring the volume to 5 ml and 5 ml of the basal medium is added.

Preparation of standard tubes: In duplicate tubes 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 ml of the working standard niacin solution (0.1 μg/ml) are taken. Sufficient water is added to bring the volume in each tube to 5 ml and then 5 ml of basal medium added to each tube. The tubes are plugged with cotton and autoclaved at 15 lb pressure for 15 min. The samples are cooled to room temperature and aseptically inoculated with one drop of inoculum and the tubes incubated at 37°C for 18 hr.

Calculation :

The growth response of the organism to niacin is found out by measuring the OD using a 66 filter. A standard graph is drawn by plotting OD against the concentration of niacin per tube in the standard series. The niacin content in the unknown tubes is determined by interpolation of the OD value on the standard graph. The niacin content per ml of the test solution is then calculated.

Reference :

Methods of Vitamin Assay, Association of Official Vitamin Chemists (Ed.), 3rd Edn, p. 169 (1966).

d. Folic Acid

Microorganism : The following 3 organisms are used to estimate one or more of the following category of folates in foodstuffs :

1. Free folates
2. Reduced folates
3. 5-Methyl THFA
4. Triglutamates

For 1, 2, 3 and 4 use *L. Casei* (A. T. C. C. No. 7469), 1 and 2 use *S. Faecalis* (A. T. C. C. No. 8043) and 2, 3 and 4 use *P. Cerevisiae* (A.T.C.C. No. 8081)

For the estimation of heptaglutamate the food extract is incubated with folic acid conjugase and the monoglutamate formed is determined by *S. Faecalis* or *L. Casei*.

Most of the folate activity in the blood is in the form of methyl THFA and hence the most common organism used for the estimation of the folate activity in blood serum is *L. Casei*.

Reagents :

1. 0.2 M Phosphate buffer, pH 7.2 : 27.23 g of KH_2PO_4 and 5.60 g of NaOH are dissolved in water and diluted to 1 l.

2. 1% Ascorbic acid : 1 g of ascorbic acid is dissolved in 20 ml water. To the solution is added about 500 - 600 mg of activated charcoal, the mixture stirred gently for 15 min, filtered and an equal volume of 0.2 M phosphate buffer added. The pH of the solution is adjusted to 6.1 and it is further diluted with an equal volume of water (to be prepared fresh on the day of assay).

3. 0.2 M Sodium phosphate buffer, pH 6.1 : Mix 85 ml of NaH_2PO_4. 2 H_2O (31.2g/l), 15 ml of Na_2 HPO_4 (28.4 g/l) and 100 ml water.

4. Folic acid stock standard : 10.0 mg of folic acid is dissolved in 100 ml of 0.8% sodium bicarbonate solution to obtain 100 μg/ml.

5. Working standard : The stock solution is diluted to get a final concentration of 1 ng/ml for assays with *S. Faecalis* and 0.1 ng/ml for assays with *L. Casei*.

6. Solution Nos. 1 to 8, 10, 12 to 14 are prepared as already described.

Basal medium for *L. Casei*,		in 100 ml
Casein hydrolysate	—	10 ml
Peptone	—	0.4 ml
DL-Tryptophan	—	10 ml
L-Cystine	—	5 ml
DL-Alanine	—	1 ml
Salt A	—	5 ml
Salt B	—	1 ml
AGU solution	—	1 ml
Xanthine	—	1 ml
Biotin (1.5 ml of stock diluted to 100 ml)	—	1 ml
Thiamine and pyridoxine solution	—	1 ml each
Riboflavin	—	1 ml
Nicotinic acid	—	2 ml
Calcium pantothenate	—	2 ml
PABA	—	0.3 ml
Glucose	—	4 g
Sodium acetate. 3 H_2O	—	6.64 g

The pH is adjusted to 6.8, the volume made upto 100 ml and the solution filtered.

Procedure :

Preparation of serum for the assay : To 0.5 ml of serum is added 8.5 ml of 0.05 M sodium phosphate buffer and 1 ml of 1% ascorbic acid and the mixture autoclaved for 10 min at 15 lb pressure. It is then cooled, filtered and again diluted 3 times (ie. dilution factor of 60).

Preparation of foodstuffs for the assay : The dry material (1.0 g) or fresh homogenised material (10 g) is taken to which is added 40 ml of 0.1 M phosphate buffer (pH 7.2) and 2.5 ml 4% ascorbic acid solution. The mixture is autoclaved for 15 min at 15 lb pressure and cooled to room temperature. The pH is adjusted to 4.5, the volume made upto 100 ml and filtered. The filtrate is used for free folate estimation after suitable dilution. For the estimation of total folate, 5 ml of filtrate, 1 ml of 0.2 M acetate buffer pH 4.5, 0.5 ml of 100 mM mercaptoethanol and 0.2 ml of human plasma are incubated

overnight at 37°C. After incubation the sample is diluted, filtered and used for assay. The sample is diluted to get a final concentration of 0.1 ng/ml for *L. Casei* and 1.0 ng/ml for *S. Faecalis* and *P. Cerevisiae*.

Take in duplicates 0.5, 1.0, 2.0 and 3.0 ml of sample. One ml of ascorbic acid (0.25%) and sufficient water are added to bring the volume to 5.0 ml followed by 5.0 ml of the basal medium.

Take in duplicates 0, 0.5, 1.0, 2.0, 3.0 and 4.0 ml of the working standard solution and 1.0 ml of 0.25% ascorbic acid and sufficient water is added to bring the level to 5.0 ml. This is followed by the addition of 5.0 ml of basal medium.

Basal medium for *S. Faecalis and P. Cerevisiae*

Reagents :

1. L. Asparagine : 3.0 g dissolved in 100 ml using ten drops of concentrated HCl for dissolving.
2. L. Cystine : 5.0 g dissolved in 100 ml of water.
3. Glutathione : 50.0 mg dissolved in 100 ml water.
4. Tween 80 : Diluted 10 times with alcohol.
5. Solution No. 1, 3 to 8 and 14 are prepared as mentioned earlier.

Basal medium		in 100 ml
Casein hydrolysate	—	10 ml
DL–Tryptophan	—	10 ml
AGU solution	—	9.5 ml
Xanthine	—	1 ml
Riboflavin	—	0.5 ml
PABA	—	2 ml
Pyridoxin and thiamine solution	—	0.8 ml each
Calcium pantothenate	—	0.8 ml
Nicotinic acid	—	0.8 ml
Biotin	—	0.2 ml
Salt A	—	1 ml
Salt B	—	1 ml
L–Asparagine	—	2 ml
L-Cystine	—	1 ml
Glutathione	—	1 ml
Tween 80	—	0.1 ml
Glucose	—	4.0 g
Sodium acetate.3 H_2O	—	6.64 g

The pH is adjusted to 6.8 and the volume made upto 100 ml and 20 mg $MnSO_4$. H_2O is added, mixed and filtered.

For *P. Cerevisiae*, 1.0 ml of folic acid (100 μg) is added to the basal medium.

For *S. Faecalis*, the stock solution is diluted to get a final concentration of 1 μg/ml folic acid and 0, 0.5, 1,2,3 and 4 ml are taken in duplicates. To this is added 1 ml of 0.25% ascorbic acid and sufficient water to bring the volume to 5 ml. This is followed by the addition of 5 ml of basal medium.

For *P. Cerevisiae*, 2 mg of DL–calcium leucoverin is dissolved in 100 ml to contain 20 μg/ml. (Stock standard solution).

Working standard : The stock solution is diluted to get a final concentration of 2 ng/ml.

Standard range (0-6 ng/ml) : 0, 0.5, 1.0, 1.5, 2.0 and 3.0 ml of the working standard are taken to which are added 1 ml of 0.25% ascorbic acid, water to make 5 ml and 5 ml of basal medium. Inoculate and then incubate at 37°C for 72 hr for *L. Casei* and titrate the acid produced against 0.1N NaOH using bromothymolblue as indicator. For *S. Faecalis* and *P. Cerevisiae* after inoculation incubate for 16-18 hr at 37°C and measure turbidimetrically at 660 nm.

Note :

The apparent analytical values for *P. Cerevisiae* activity are to be halved to obtain the true *P. Cerevisiae* activity.

References :

1. Biochem. J. **50**, 326 (1952).
2. J. Clin. Invest. **40**, 81 (1961).

e. Pyridoxine

Principle :

Total vitamin B_6 is estimated microbiologically using *Saccharomyces Carlsbergensis* as assay organism.

Reagents :

1. 1 g of KH_2PO_4, 0.85 g KCl, 0.25 g $CaCl_2$, 0.25 g Mg SO_4 and 0.005 g $MnSO_4$ are dissolved in water with warming. 0.005 g $FeCl_3$ is added. If a precipitate is formed dissolve it by adding a few drops of 1N HCl. The volume is made to 100 ml.

2. Casein hydrolysate : (given under preparation of common solutions).

3. 4 mg of D–biotin are dissolved in 100 ml distilled water with warming if necessary.

4. 5 mg Calcium pantothenate, 50 mg mesoinositol and 5 mg niacin are dissolved in 100 ml distilled water with warming.

5. 5 mg Thiamine – HCl are dissolved in 10 ml distilled water.

6. 10 g Potassium citrate and 2 g citric acid are dissolved in 100 ml distilled water.

Basal medium

10	ml of reagent	—	1
0.8	ml of reagent	—	2
0.4	ml of reagent	—	3
10	ml of reagent	—	4
0.25	ml of reagent	—	5
10	ml of reagent	—	6

The above solutions are mixed ; pH is adjusted to 5.5 and made upto 100 ml. To 100 ml of this medium 60 ml of glucose solution (100 g in 600 ml water) is added.

Medium for stock culture

Malt extract	—	3.3 g (Difco)
Glucose	—	1.0 g
Yeast extract	—	0.3 g (Difco)
Peptone	—	0.5 g (Difco)
Agar	—	2.1 g (Difco)

The above ingredients are dissolved in 100 ml water by heating. Portions of 8 ml are dispensed nto test tubes while the solution is still hot. The tubes are plugged with cotton and sterilised at 15 lb or 15 min. Tubes are allowed to cool in slanting position. *Saccharomyces Carlsbergensis* is subcultured very fortnight in slope cultures.

Inoculum : A freshly grown agar slope culture (after 20 hr incubation) is washed with 10 ml 0.9% saline and transferred under sterile conditions into a sterile tube. As this solution is very rich in rganisms, a suitably diluted inoculum of 15% turbidity is prepared and is used in inoculating the standard nd sample solutions.

15% Turbidity = 85% T = 0.071 OD = 35.5 Klett readings at 660 nm.

Stock standard solution : 50 mg of pyridoxine is dissolved in 100 ml distilled water.

Working standard solution (prepared daily) : One ml of the stock standard is diluted to 100 ml nd will contain 5 μg/ml. This is further diluted 100 fold which now contains 50 μg/ml. Dispense tandards containing 0, 5, 10, 15, 20, 25, 30, 40 and 50 ng of pyridoxine into 50 ml flasks and make pto 1 ml.

Procedure :

Preparation of samples

Foods of vegetable origin : About 10 g of the dry food is powdered. In the case of vegetables nd fruits, a representative sample of cut material is prepared. Two g of fresh material or dry powder s suspended in 20 ml water, 20 ml of 0.5 N H_2SO_4 are added and autoclaved for 4 hr at 15 lb pressure. The mixture is cooled and pH is adjusted to 5.5, diluted to 100 ml and filtered. From this ltrate suitable aliquots are taken in duplicate at 3 levels for vitamin B_6 assay.

Flesh foods : Aqueous homogenates (10%) of the foods are prepared in a waring blendor. One nl of the homogenate is diluted to 5 ml with water, 5 ml of 1N HCl is added and autoclaved for 4 hr t 15 lb pressure. The mixture is cooled, pH is adjusted to 5.5 and diluted to 50 ml and filtered. Suitable aliquots in duplicate at 3 levels are taken for vitamin B_6 assay.

Urine : To 10 ml of a 24 hr urine sample (well mixed), 10 ml 1N HCl is added and autoclaved or 4 hr at 15 lb pressure, cooled and pH adjusted to about 5.5 and diluted to either 50 or 100 ml and ltered. Aliquots of 0.5 and 1.0 ml are taken in duplicate for vitamin B_6 assay. Vitamin B_6 values are xpressed as mg of vitamin B_6 per 24 hr urine sample.

Blood : To 5 ml of whole blood, 5 ml of 1N HCl is added and autoclaved for 4 hr at 15 lb pressure, cooled and pH is adjusted to 5.5. It is then filtered through Whatman No. 42 filter paper. The recipitate is washed twice with small amounts of water. The volume of the filtrate and washings is ade upto 25 ml. The filtrate is tested for any precipitate by heating in the autoclave at 15 lb pressure for 15 min. One ml aliquots in duplicate are taken for assay of vitamin B_6. Concentration of itamin B_6 in blood is expressed as μg vitamin B_6 per 100 ml blood.

Inoculation and incubation : The standards or samples are dispensed into 50 ml conical flasks and volume made to 1.0 ml. Eight ml of the basal medium are added and sterilised for 12 min at 12 lb pressure. One ml of the inoculum (turbidity 15%) is added under sterile conditions and incubated at 37°C for 20 hr.

The turbidity is measured in a Klett-Summerson colorimeter with 66 filter.

Reference :

Vitamin assay tested methods. R. Strohecker and H. M. Henning. translated by D. D. Libman p. 144 (1965).

f. Cyanocobalamin

Microorganism : *Euglena Gracilis*

Reagents :

Basal medium (double strength) : The following are weighed out and mixed well in a clean mortar and pestle.

*	Metal mixture	—	22 mg
	Calcium carbonate	—	80 mg
+	Thiamine sucrose mixture	—	250 mg
	KH_2PO_4	—	300 mg
	$Mg\ SO_4.\ 7\ H_2O$	—	400 mg
	Succinic acid	—	466 mg
	Ammonium bicarbonate	—	624 mg
	DL–Malic acid	—	1.0 g
	DL–Aspartic acid	—	2.0 g
	Glycine	—	2.5 g
	L–Glutamic acid	—	3.0 g
	Sucrose	—	15.0 g

* Metal mixure : This is prepared by mixing the following

$VaSO_4.\ H_2O$	—	30 mg
$CuSO_4.\ 5H_2O$	—	310 mg
$CoSO_4.\ 7H_2O$	—	480 mg
H_3BO_3	—	560 mg
$(NH_4)_6\ Mo_7O_{24}.\ 4H_2O$	—	630 mg
$MnSO_4.\ H_2O$	—	1.55 g
$ZnSO_4.\ 7H_2O$	—	4.40 g
$FeSO_4\ (NH_4)_2\ SO_4.\ 6H_2O$	—	14.00 g

\+ Thiamine sucrose mixture : Prepared by mixing 12 mg thiamine and 5.0 g sucrose.

The dry mixure of basal medium (5.13 g) is dissolved in about 80 ml of distilled water by gentle heating and the volume made upto 100 ml.

Maintenance of stock culture : The culture medium is prepared by the addition of 400 mg tryptone and 1 ml of vitamin B_{12} solution (containing 10 mg vitamin B_{12} per ml) to 100 ml double strength basal medium and the volume made upto 200 ml. Ten ml of this is dispensed in test tubes plugged with cottonwool and sterilised by autoclaving at 10 lb pressure for 10 min. The tubes thus prepared could be kept for a month. Three or four drops of a suspension of the organism are added to

or 3 tubes aseptically and they are incubated in an illuminated water bath kept at 25–30°C. Full rowth takes place within 6–8 days. Similarly, the organism is subcultured every week.

Preparation of the inoculum : The method is similar to the one for the stock culture except that yptone is omitted in the preparation of these tubes.

Procedure :

Extraction of B_{12} from the sample, Serum : To 1.0 ml of serum is added 1 ml 0.1 M sodium cetate buffer (pH 4.6), two drops of 0.1 % NaCN solution and 1 ml of distilled water. The contents are ixed and steamed for 30 min, cooled and made upto 10 ml and filtered. A known volume of this ltrate is taken and the pH adjusted to 3.5 using 0.1 N HCl. This is further diluted so as to get an pproximate concentration of 10 μg/ml.

Milk : To 1 ml of milk is added 1 ml 0.1 M sodium acetate buffer, pH 4:6, two drops of 0.1% aCN and 50 mg papain. The contents are mixed incubated at 60°C for 1 hr, at the end of which it is eamed for 5 min and then the procedure is followed as for serum.

Standard tubes : To a series of tubes in duplicate is added 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 0 ml of a standard solution containing 20 μg of vitamin B_{12} and the volume is made upto 2 ml with stilled water.

Sample tubes : The diluted sample extract is added into 4 sets of tubes 0.5, 1.0, 1.5 and 2.0 ml spectively and the volume made upto 2.0 ml with distilled water.

To each tube is added 2 ml of double strength medium, the tubes covered with aluminium caps d autoclaved at 10 lb pressure for 10 min. After cooling the tubes are seeded with a washed sus- nsion of *Euglena Gracilis* and incubated in an illuminated water bath kept at 25–30 C. The tubes are ken out after 6 days, steamed for about 3 min and diluted with 10 ml distilled water. The turbidity measured in Klett–Summerson colorimeter using 600 nm filter. The blank is adjusted against distilled ater. A standard graph is drawn by plotting the turbidity against the concentration of vitamin B_{12} in e standard tubes and the concentration of vitamin B_{12} in the test solution is calculated using the aph.

Calculation :

Amount of vitamin B_{12} = average vitamin B_{12} content per ml of test solution x total dilution.

Note :

The vitamin B_{12} activity remains unaltered for a month if the samples are kept frozen. If it is cessary to preserve the samples for longer periods, they are freeze-dried and kept in sealed ampoules the cold.

References :

1. J. Protozool. 3, 101 (1956).
2. Brit. J. Nutr. 8, 340 (1954).

CHEMICAL METHODS

Thiamine

(i) Food

Principle :

This is based on the oxidation of thiamine to thiochrome, which fluoresces in UV light. Under ndard conditions and in the absence of other fluorescing substances, the fluorescence is proportional

to the thiochrome present and hence to the thiamine originally in solution.

Reagents :

1. Preparation of vitamin extract : About 10-20 g of the well-mixed ground food sample is taken and treated with 100 ml of acetate buffer, pH 4.0–4.2, (made by mixing 30 ml of 1 M sodium acetate solution with 70 ml of 1 N acetic acid and making upto 500 ml) and the mixture blended in a waring blender, if necessary, for thorough mixing. To the slurry, 5 ml of enzyme suspension containing 150 mg of taka-diastase and 75 mg of papain in 5 ml of acetate buffer is added. An enzyme blank containing the buffer and enzymes but without the sample is also run simultaneously. A few drops of toluene are added and the mixture is incubated overnight at 37°C. The enzymes are inactivated by heating in a water bath at about 80°C for 5 – 10 min, the solution cooled, the mixture centrifuged and the clear centrifugate collected. Aliquots of this solution are used for estimating thiamine and riboflavin.

2. Basic lead acetate solution : Lead acetate, 180 g, is dissolved in about 700 ml of distilled water and the solution boiled. To the boiling solution is added in small quantities lead oxide (litharge) 180 g and the solution boiled for 30 min. It is then cooled, made upto 1 l with distilled water and filtered.

3. Pure thiamine solution : 25 mg of pure thiamine dissolved in 250 ml of 0.01 N HCl. 1 ml of this stock solution is diluted to 100 ml to get a working standard containing 1 μg/ml.

Procedure :

Twenty ml of the vitamin extract is treated with 10 ml of basic lead acetate solution, mixed well and centrifuged. A suitable aliquot of the supernatant (20 or 25 ml) is treated in another centrifuge tube with 3 ml of 30% H_2SO_4 and 12 ml of water (or enough water to make the total volume to 40 ml), mixed well and centrifuged. Two ml of 40 % NaOH is added to 3, 100 ml separating funnels (one of them serving as a blank, the other as the experimental and the third as recovery). Add 0.8 ml of 2% potassium ferricyanide solution to the experimental and recovery, shake well and 10 ml of thiamine extract is added to the separating funnels. To the recovery, 1 μg of pure thiamine standard solution is added and the contents allowed to stand for 2 min after shaking. Then 15 ml of isobutyl alcohol (water saturated) is added and shaken for 2 min. The aqueous layer is rejected. If the alcohol layer is turbid, 1 ml of ethyl alcohol is added to clear the solution. The fluorescence of the isobutyl alcohol is measured in a fluorimeter using the appropriate filters. The calculation is done after making due allowance for the dilution and the blank.

Calculation :

$$\mu\text{g of Thiamine/100 g of sample} = \frac{\text{Experimental - Blank}}{\text{Recovery - Experimental}} \times \text{Dilutions} \times \frac{100}{\text{Wt of the sample}}$$

References :

1. J. Am. Chem. Soc. **61**, 179 (1939).
2. J. Biol. Chem. **60**, 361 (1945).

(ii) Urine

Principle :

Thiamine is absorbed from urine in acid solution by means of activated decalso. It is eluted with

concentrated potassium chloride and is converted to thiochrome by potassium ferricyanide in alkaline solution. Thiochrome is extracted into isobutyl alcohol and measured fluorimetrically.

Reagents:

1. 25% KCl in 0.1 N HCl (heat and filter).
2. Bromocresol green solution: 0.4% in absolute ethyl alcohol.
3. 2.5 M Sodium acetate.
4. Decalso: Wash 60 to 70 mesh decalso three times with 3% glacial acetic acid and once with 25% KCl and wash again with 3% acetic acid and then several times with distilled water. Each washing consists of stirring decalso for 15 min in the solution allowing to settle and then decanting. Store in wet state in a stoppered bottle.
5. 1N NaOH.
6. 0.5% Acetic acid.
7. 1% Potassium ferricyanide. This solution is stable for 6 months if kept in a dark bottle in cold.
8. 15% Potassium hydroxide. This solution is stable if kept in cold.
9. Oxidising reagent: One part of 10% potassium ferricyanide mixed with 9 parts of 15% KOH (prepare fresh daily).
10. Stock thiamine solution: Dry thiamine hydrochloride for 2 hr at 105°C and store in a desiccator over P_2O_5 for atleast 24 hr before use. In a 500 ml volumetric flask dissolve 50 mg of thiamine hydrochloride in 200 ml of 95% ethyl alcohol, add 5 ml of 0.1 N HCl and bring to volume with distilled water (Stable for 6 months if kept in cold). Dilute exactly 5 ml of thiamine stock solution to 100 ml with distilled water (5 μg/ml).

Working standard: Transfer 4 ml of this solution to a flask containing 75 ml of 0.1 N H_2SO_4 and 5 ml of sodium acetate solution. Adjust to 100 ml with water. The final concentration of this solution will be 0.2 μg/ml (Prepare daily).

Procedure:

Place a small piece of glass wool at the tip of the absorbing columns. Fill the columns with activated decalso. Uniform filling is accomplished by first filling the tube with water, then adding the decalso and allowing it to settle with gravity.

Wash the column with 3 ml of 0.5 % acetic acid. Allow to drain in waste pan.

Place a 10 ml aliquot of urine in a 50 ml beaker, add 2 drops of bromocresol green and neutralise to a light yellowish green colour with 1 N NaOH or 1 N H_2SO_4 or adjust the pH to 3.5.

Pour the sample on to the column and allow it to drain by gravity into the waste pan.

Rinse the beakers once with 10 ml of hot distilled water and pour these washings through the column. Then wash the columns twice more with 10 ml of hot distilled water. Each washing should be permitted to drain into the column before another is added.

Place 25 ml volumetric flask under each column and elute successively with 10, 10, 5 ml of 25% KCl (boiling). Each aliquot should be permitted to drain into the column before another is added. The eluates are stable if kept in cold for at least one week.

One can intercept the procedure at this point.

Remove two 5 ml aliquots from each flask into two reaction tubes, one for unknown and the other for blank. To the unknown tube add 0.7 ml of oxidising reagent and mix. Extract with 10 ml of isobutyl alcohol. For blank tube add 0.7 ml of KOH (1 part of H_2O and 9 parts of 15% KOH), extract with 10 ml of isobutyl alcohol. This reaction of oxidation and extraction should be done within 1 min. Extraction for 45 sec is enough. Remove the top layer (isobutyl alcohol) add a pinch of anhydrous Na_2SO_4 and take the readings in a flourimeter, (EEL flourimeter filters, primary = OX1 and secondary = OB10; Farrand flourimeter, excitation wavelength 370 nm and fluorescence wavelength 445 nm).

Reference :

Manual for nutrition surveys, ICNND, p. 136 (1963).

b. Riboflavin

(i) Food :

Principle :

The native fluorescence of riboflavin in neutral pH is used in the chemical estimation of this vitamin.

Reagents :

1. Riboflavin standard solution : 25 mg of riboflavin is dissolved in 300 to 400 ml of water, adding 1.2 ml of glacial acetic acid and warming at a low temperature to aid solution. After the riboflavin is dissolved, the solution is cooled and made upto 1 l. This stock solution has a concentration of 25 μg per ml. Two ml of this stock solution is diluted to 50 ml to give a working standard of 1 μg per ml.

2. 4% Potassium permanganate.

3. 1 : 1 H_2O_2-water mixture.

Procedure :

To about 25 ml of the vitamin extract, one or two drops of caprylic alcohol are added, followed by 3 ml of freshly prepared 4% potassium permanganate solution. The mixture is stirred well and within 2 min 3 ml of 1 : 1 H_2O_2-water solution is added to discharge the permangante colour and the pH adjusted to 7.0 with NaOH. The volume is made upto 35 ml and the solution is filtered and the fluorescence of the filtrate is measured in a fluorimeter using the appropriate filters.

Calculation :

The fluorescence of a known aliquot of the filtrate is — A

1 μg of riboflavin is added and the reading noted—B

A small pinch of sodium hydrosulphite is added to destroy the riboflavin only and the reading recorded —C

Then the quantity of riboflavin in the solution taken for fluorimetry $= \frac{A - C}{B - A} \times 1\,\mu g.$

From this, the riboflavin content of the sample is calculated making due allowance for dilution and enzyme blanks etc.

(ii) Blood

Principle :

The fluorescence intensity of FAD is only about 9–14% of FMN and free riboflavin in high salt solutions. Hence, the measurement of flourescence before and after hydrolysis gives an estimate of FAD and total riboflavin.

Reagents:

1. 20% TCA in glass distilled water.
2. 4 M Potassium phosphate (dibasic).
3. Stock riboflavin standard solution 10 mg/100ml.

Procedure :

Extraction of flavin (If FAD has to be estimated, the extraction should be done at 5°C).

Take 0.1 ml of erythrocyte haemolysate (prepared by mixing washed erythrocytes with equal volume of water and freezing for 24–48 hr or a 2% homogenate of liver and make the volume to 1 ml with glass distilled water. Add 1 ml of 20% TCA and mix thoroughly to extract the flavins. Centrifuge and use the supernatant for fluorimetric estimation of free riboflavin. To an aliquot of the supernatant add half the volume of 4 M potassium phosphate buffer. Immediately transfer to a microcuvette and centrifuge at 3000 rpm. Measure the fluorescence at 530 nm with excitation set at 470 nm (F_1). Add a few crystals of solid sodium hydrosulphite (with the aid of a thin glass rod, moistened at the tip) to the cuvettes and mix. Centrifuge for 30 sec and again measure the fluourescence (Blank).

To release free riboflavin from FMN and FAD, incubate an aliquot of the supernatant at 37°C overnight and proceed through all the steps given earlier for total riboflavin. F_2 corresponds to total flavins in the extract.

Prepare a series of standards (10-40 mg) and proceed similarly.

Prepare a standard graph by plotting the fluorescence value for different levels of standards,

Calculation :

Read the concentration of riboflavin in the sample using the standard graph. Express FAD concentration also in terms of riboflavin.

$$\text{FAD} = \frac{\text{riboflavin before hydrolysis } (F_1) - \text{riboflavin after hydrolysis } (F_2)}{0.86}$$

Reference :

J. Biol. Chem. 180, 755 (1949).

c. Flavin adenine dinucleotide

Principle :

The enzyme D-amino acid oxidase (DAAO) requires FAD as a cofactor. The enzyme activity in the presence of excess apo-DAAO and limiting amounts of FAD is directly proportional to the concentration of FAD in the reaction mixture.

Reagents :

1. Dextran coated charcoal : Mix 1g of dextran T—80 with 10 g of activated charcoal.

2. 0.033 M Pyrophosphate buffer, pH 8.3 : Dissolve 3.62 g tetrasodium pyrophosphate in about 200 ml glass distilled water. Adjust pH to 8.3 and make up the volume to 250 ml.

3. 300 mM D-Alanine : Dissolve 268.6 mg D-alanine in 10 ml glas distilled water.

4. 20% TCA in glass distilled water.

5. 2,4-Dinitrophenylhydrazine (DNP) : Dissolve 10mg DNP in 100ml 1 N HCl.

6. 2 N NaOH.

7. Crude apo-DAAO from kidney : Decapitate an adult rat and remove the kidney. Prepare a 20% homogenate in ice cold glass distilled water. Dilute it with 10 volumes of glass distilled water and allow it to stand at 0°C for 15 min. Mix dextran coated charcoal (5 mg/ml) and centrifuge immediately at 4°C and 1200 g for 30 min. Take the supernatant for assay (Prepare fresh).

Procedure :

Preparation of sample :

Liver and kidney : Prepare a 10% homogenate of liver or kidney in glass distilled water. Heat the homogenate in a water bath at 80°C for 15 min with occasional shaking. Cool and centrifuge at 3500 rpm for 15 min. Use the supernatant for FAD estimation.

RBC : Centrifuge heparinised blood at 2500 rpm for 10 min. Remove plasma and the buffy coat. Wash the cells twice with saline. Haemolyse 1 ml of packed red cells with 4 ml glass distilled water. Heat the haemolysate at 80°C in water bath for 15 min. Cool and filter. Wash the precipitate with 1 ml of glass distilled water and pool both the filtrates. Add 3 ml of water saturated phenol to the filtrate, stir well and centrifuge to separate the layers. Transfer the phenol layer to a glass stoppered tube and mix well with 10 ml of distilled ether. Centrifuge the tubes at 2500 rpm for 5 min. Remove the ether layer with phenol. Wash with ether twice to remove phenol completely. Remove residual ether by warming in a water bath at 50°C. Use the concentrated extract for FAD estimation.

To 1 ml of pyrophosphate buffer add 0.5 ml of apo-DAAO, 0.1 ml tissue extract or standard FAD solution containing 100-500 ng FAD and 0.3 ml glass distilled water. Incubate the reaction mixture at 37°C for 10 min in a shaking water bath. Start the reaction by adding 0.1 ml D-alanine. Pass oxygen into each tube for 10 sec and close the tubes with rubber stoppers. Incubate the tubes for 1 hr at 37°C in a shaking water bath. Stop the reaction by adding 0.5 ml of 20% TCA. Centrifuge the tubes at 3000 rpm for 10 min and estimate the pyruvate formed in the supernatant. To 1 ml of the supernatant add 0.25 ml of DNP solution and keep for 5 min. Add 2.5 ml of 2 N NaOH to all the tubes. Measure the absorbance at 540 nm.

Read the OD value obtained against FAD standard graph.

Reference :

Indian J. Med. Res. **73**, 404 (1981).

1. Pyridoxal phosphate

Principle :

The assay is based on the enzymatic decarboxylation of tyrosine by pyridoxal phosphate (PLP) ependent enzyme tyrosine decarboxylase. In presence of excess of apoenzyme and substrate and mited amount of PLP the rate of reaction is directly proportional to the amount of PLP in the incuba- on mixture. With L-tyrosine-1 - ^{14}C as the substrate measure the PLP dependent formation of radio- ctive CO_2.

$$\text{L-Tyrosine-1-}^{14}\text{C} \xrightarrow[\text{Tyrosine decarboxylase apoenzyme}]{\text{PLP}} \text{Tyramine} + {}^{14}CO_2$$

Reagents :

1. Saturated solution of L-tyrosine (100 g/100 ml) in 1.0 M potassium acetate buffer pH 6.0.
2. L-Tyrosine in 0.15 N HCl : 90.6 mg/10 ml.
3. L-Tyrosine-1-^{14}C : Take 80 ml of reagent 1 and 10 ml of reagent 2 in a beaker. Add 10 μCi f L-tyrosine-1-^{14}C (specific activity 19.1 mCi/mmole). Adjust the pH to 5.5 with 1 N HCl. Filter nrough glass wool and make up the volume to 100 ml with water.
4. PLP standard : Dissolve 2 mg of PLP in 100 ml of glass distilled water. Dilute 0.1 ml (2 μg) f the above solution to 100 ml with potassium acetate buffer, 0.01 M pH 5.5 containing 0.005 M DTA. (Prepare standard fresh every day).
5. Toluene sctnillation fluid (Refer Appendix).
6. Tyrosine apodecarboxylase : Preapare just before use a suspension of 20 mg of tyrosine podecarboxylase (acetone powder) in 1 ml of potassium acetate buffer, 0.01 M pH 5.5.
7. Extraction of PLP from plasma : Add 0.1 ml of 75% TCA to 1 ml of plasma, mix and entrifuge at 2000 g for 10 min. To remove TCA from the extract, shake the supernatant 4 to 6 times vith peroxide free ether saturated with water. After extraction assay should be done on the same day.

Procedure :

Pipette 0.1 ml of 0.01 M acetate buffer, 0.05 to 0.2 ml of standard or sample and 0.1 ml of apoen- yme suspension to the main chamber of a Warburg flask. In case of enzyme blank, pipette 0.2 ml of vater instead of sample. In each case make up the volume to 0.4 ml with water. Place 0.6 ml of -tyrosine-1-^{14}C in the side arm of the Warburg flask. Keep small cups containing 0.1 ml of hyamine ydroxide in the central well. Stopper the Warburg flasks and incubate at 37°C for 15 min with gentle haking. Start the reaction by adding the substrate from the side arm. Incubate the flasks with entle shaking for 8 min. Stop the reaction by adding 0.1 ml of 10% TCA to the main chamber of the ask. Stopper the flasks immediately and shake it again at room temperature for 30 min. After 30 min, emove the cups and drop it into 5 ml toluene scintillator in a vial and mix it well. Take the radioactive ounts in a scintillation counter.

Plot dpm against the concentration of standard PLP and use this standard graph to determine PLP unknown samples.

Note :

Extraction of PLP from the sample and assay is carried out in dim light. Same procedure can be llowed for the estmation of PLP in other tissues also. Plasma can be stored at - 20°C for 3 days.

Reference :

Methods in Enzymology. Vol. XVIII, p. 509 (1970).

e. 4 - Pyridoxic acid

Principle :

4 – Pyridoxic acid is estimated fluorimetrically in urine after removing the impurities present using ion exchange resins.

Reagents :

1. 0.05, 1.0, 2.0 and 5.0 N HCl
2. 5 N NaOH
3. 1.5 N NH_4OH
4. 1% Borax (aqueous)

5. Pyridoxic acid standard solution : Ten mg 4 - pyridoxic acid is dissolved in distilled water by dropwise addition (5 to 6 drops) of 0.1 N NaOH and diluted to 100 ml. This contains 100 μg of 4-pyridoxic acid per ml. From this solution 0.3 ml is added to 2 N HCl and volume is made upto 80 ml with 2 N HCl.

6. Ion exchange resins : Dowex-1 (Cl^-) 8% cross linkage and 200-400 mesh and Dowex-50 (H^+) 12% cross linkage and 100-200 mesh. A slurry of each resin is pipetted into the columns to form a packed layer of 3 cm long. The Dowex-1 column is washed with 50 ml of 2N HCl followed by 50 ml of water. The Dowex-50 column is washed successively with 50 ml of 5 N HCl, 2 N HCl and water. Chromatographic columns are made from tubes of 1.2 cm diameter and 30 cm long.

Procedure :

Elution of 4-pyridoxic acid : Urine 100 ml from a 24 hr collection is taken and pH is adjusted to 10.6 by adding a few drops of saturated NaOH and filtered through Whatman No. 42 filter paper. Twenty to 30 ml of this filtrate is taken in duplicate in 50 ml beakers. To one, 0.3 ml of standard solution containing 30 μg pyridoxic acid is added for determination of recovery. Add 1.5 ml of 1.5 N NH_4OH to both the beakers. The volume is made upto 40 ml and the solution is loaded on the Dowex-I (Cl^-) column. Beakers are rinsed once with 20 ml water and this 20 ml is also added on to the column. 4–Pyridoxic acid is retained on the column which is eluted with 80 ml of 0.05 N HCl. This eluate (80 ml, 0.05 N HCl) is loaded on the Dowex-50 (H^+) column. Dowex-50 column is washed with 20 ml water and 4-pyridoxic acid is eluted with 80 ml of 2 N HCl.

Estimation of 4–pyridoxic acid : Eight ml of standard solution (in 2 N HCl) and 8 ml of urinary eluates (in 2 N HCl) are taken in two boiling tubes. Four ml of 5 N NaOH is added and the tubes heated in a boiling water bath for about 20 min and cooled. Excess alkali is neutralised by adding 4 ml of 1 N HCl and further 4 ml of 5 N HCl (to make the final normality 1N) is added and heated in a boiling water bath for 25 min. To this, 4 ml of 5 N NaOH is added just to neutralise the acid present and diluted to 30 ml. Immediately after making the solution upto 30 ml 1, 2, 3 and 4 ml aliquots from the standard solution are added to tubes containing 9, 8, 7 and 6 ml of 1% borax solution and mixed well. Similarly 2 and 4 ml aliquots (after diluting to 30 ml) of sample solutions are added to tubes containing 8 and 6 ml of 1% borax solution. Fluorescence is measured using excitation wavelength of 365 nm and fluorescence wavelength of 440 nm with 3-73 filter in the measuring place in a Farrand spectro fluorimeter.

Calculation :

The 4-pyridoxic acid is expressed as mg per 24 hr urine sample.

eg : A 20 ml urine sample obtained after filtration is made to 40 ml which is added on the owex-1 column and the eluate of this column on to the Dowex-50 column, finally eluted from owex-50 column by 80 ml of 2 N HCl.

Eight ml aliquot from the final eluate is processed as described in the procedure and made upto 0 ml from which 4 ml aliquot is pipetted out into 6 ml of the borax solution and the fluorescence ieasured. Suppose this fluorescence reading corresponded to X μg in the standard graph.

$$\text{then 4-pyridoxic acid/24 hr urine sample} = \frac{X}{4} \times \frac{3}{8} \times \frac{80}{20} \times \frac{V}{1000} \text{ mg}$$

where V = volume of the 24 hr urine sample.

Reference :

J. Biol. Chem. **233**, 691, (1958).

Niacin

Principle :

Nicotinic acid reacts with cyanogen bromide and aromatic amines like aniline, metol etc., to give a ellow coloured compound, which is measured colorimetrically.

Reagents :

1. Stock calcium hydroxide suspension : 250 g of calcium hydroxide in 1 l of water.
2. Working calcium hydroxide suspension : Reagent 1 is suitably diluted to give 2 N calcium ydroxide. This is checked by titration.
3. Zinc sulphate solution : 80 g of $ZnSO_4.7\ H_2O$ dissolved in 100 ml of water.
4. Phosphate solution : 5 g of KH_2FO_4 in 100 ml of water.
5. Niacin standard : 500 mg dissolved in water and 5 ml of 10 N H_2SO_4 is added and made upto 00 ml.
6. Cyanogen bromide solution : 27 ml of bromine kept in a conical flask immersed in ice is eutralised with 10% solution of freshly prepared NaCN using a burette till the solution becomes colour-ss. Ten extra drops of NaCN are added and the solution made upto 1 l.
7. Buffered CNBr: 500 ml of CNBr soultion (6) are added to 25 g of KH_2PO_4 in a 500 ml lumetric flask, dissolved and kept in the cold.
8. 8% metol in 0.5N HCl.

Procedure :

Digestion of sample : Two g of the finely powdered sample is suspended in 10 ml of calcium droxide and the volume made upto 50 ml.

Sample, recovery and blank tubes are similarly prepared and all of them are heated in a boiling ater bath for 90 min. The contents are stirred frequently in the early stages. After 90 min the tubes e cooled in a bath of cold water and the solution made up again to 50 ml and centrifuged for 20 min 2000 rpm. A 25 ml aliquot of the centrifugate is transferred to another calibrated tube and acidified

with 0.5 ml of 8 N HCl and then 2 ml of $ZnSO_4$ solution 2 drops of caprylic alcohol and 2 drops of phenolphthalein are added. Add 4N NaOH with cooling till Zn $(OH)_2$ is formed. Then 1 N NaOH is added until the mixture turns pink. Then add 5N H_2SO_4 till the pink colour just disappears after which the volume is made upto 50 ml, centrifuged and aliquots of centrifugate used to develop the colour.

Development of colour : Each solution is taken in 4 tubes (A, B, C, D) to allow for corrections due to amine, CNBr and other interfering colours.

	A ml	B ml	C ml	D ml
Filtrate	5	5	5	5
CNBr reagent	2	—	2	—
5% KH_2PO_4	—	2	—	2
8% metol in 0.5 N HCl	3	3	—	—
0.5 N HCl	—	—	3	3

The yellow colour developed is read at 400 nm in a colorimeter.

Calculation :

The colour due to the 5 ml filtrate (0.1 g sample) alone $= A - (B + C - D) = a$.

The colour in the recovery tube (0.1 g sample $+ 5\mu$g niacin) $= A_R - (B_R + C_R - D_R) = b$.

$\therefore b - a = 5\mu$g of niacin or 1μg of niacin $= \frac{b-a}{5}$ and 0.1 g of substance contains $\frac{5a}{b-a}$

$$\therefore \% \text{ niacin} = \frac{5a}{(b-a)} \times \frac{100}{0.1}$$

Reference :

Arch. Biochem. **26**, 361 (1950).

g. Nicotinamide Nucleotides

Principle :

The principle involves the alkali-ketone condensation of the nicotinamide nucleotides to form a highly fluorescent product which is measured in a spectrofluorimeter.

Reagents :

1. 10% (W/V) TCA
2. 5 N HCl
3. 5 N NaOH
4. Ethylmethylketone
5. 20% KH_2PO_4 solution
6. Standard solution of NAD (1.0μg/ml).

Procedure :

An accurately weighed piece of liver is homogenised with ice cold 10% TCA to yield a 10% homogenate.

Oxidation of ascorbic acid : To the above solution is added 2-3 drops of bromine from a dropper. It is shaken gently until the solution is slightly yellowish, decanted from excess bromine and air or nitrogen (saturated with water) is passed through the solution until the dissolved bromine is expelled. (If the solution does not become colourless, trace of residual bromine will be taken care of by thiourea added later on).

To a 5 ml aliquot of oxidised extract is added 10 ml of 2% thiourea in 5% HPO_3 and 5ml of 5% HPO_3 and mixed thoroughly. It is then processed as given below.

Formation of osazone

1. 4 ml aliquots of the sample are pipetted into each of 3 tubes.
2. One tube of each sample is set aside to serve as a blank.
3. To each of the remaining tubes is added 1 ml of 2% 2,4 – dinitrophenylhydrazine reagent.
4. All the tubes are placed in an incubator at 37°C for 6 hr.
5. At the end of 6 hr the tubes are removed from the incubator and placed in an ice bath.

While the tubes are in the ice bath 5 ml of 85% H_2SO_4 is added slowely to each tube. The addition should be made dropwise and should take atleast 1 min. (Addition of 85% H_2SO_4 fast or at room temperature may make the solutions dark).

With the tubes still in the ice bath, 1 ml of 2% 2,4 – dinitrophenylhydrazine reagent is added to the blank. All the 3 tubes ere shaken to mix the contents.

The tubes from the ice bath are removed and allowed to stand 30 min at room temperature. The colour is read in a photoelectric colorimeter at 540 nm.

A calibration chart is prepared by using standard ascorbic acid solutions containing 1, 2, 4, 5, 8 and 10 μ g/ml. ODs are plotted against concentration of ascorbic acid (μ g/ml).

Calculations :

The total ascorbic acid content of each aliquot is calculated according to the formula,

$$\text{mg total ascorbic acid/100 g} = \frac{R}{W} \times \frac{100}{1000}$$, where R = mg of total ascorbic acid per ml of solution (diluted sample) obtained by reading from the calibration chart the value corresponding to the readings and W = g of sample/ml of diluted solution.

Note :

Fresh samples are bought from the market and analysed immediately. If the foodstuff has to be preserved it should be kept in cold storage.

A 10% concentration of $SnCl_2$ in the mixture protects ascorbic acid from oxidation and leaves dehydroascorbic acid and diketogulonic acid unaltered. After the sample is macerated, the contents are transferred to a graduated cylinder and made upto 200 ml with 5% HPO_3 and filtered.

References :

1. Methods of Vitamin Assay. Association of Vitamin Chemists Inc. (Ed.), Interscience Publishers, p. 159 (1947).
2. J. Biol. Chem. **152**, 511 (1944).

(ii) Plasma

Principle :

This method measures the total of L-ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid and is based upon the measurement of the colour of the product formed by the coupling of 2, 4-dinitrophenylhydrazine with the ketonic group of oxidised ascorbic acid.

Reagents :

1. 2, 4-Dinitrophenylhydrazine : 2 g of 2, 4-dinitrophenylhydrazine is dissolved to make upto 100 ml with approximately 9 N H_2SO_4 and filtered.

2. 6% TCA

3. Acid washed norit : 200 g of norit is suspended in 1 l of 10% HCl, heated to boiling and filtered under suction. The cake is removed and stirred with 1 l of water and refiltered. This procedure is repeated until the washings give a negative test for ferric ions. The norit is then dried overnight at 110 - 120°C.

4. 85% H_2SO_4

5. Thiourea : Ten g of thiourea dissolved in 100 ml of 50% (V/V) aqueous alcohol.

Procedure :

To 2 ml of plasma, 6 ml of TCA is added in a centrifuge tube, stirred and allowed to stand for 5 min and centrifuged.

The supernatant solution is shaken vigorously with about 300 mg of acid washed norit and filtered (norit clarifies the solution and oxidises ascorbic acid to dehydroascorbic acid in the presence of TCA). To a test tube containing 1.6 ml of norit filtrate, is added one drop of thiourea solution and 0,4 ml of 2,4 - dinitrophenylhydrazine reagent. The tube is incubated at 37°C for 3 hr and then placed in an ice water bath along with a blank tube containing 1.6 ml of norit filtrate and one drop of thiourea solution. Colour is developed by adding dropwise 2 ml 85% H_2SO_4 while stirring. Finally 0.4 ml of 2,4-dinitrophenylhydrazine reagent is added to the blank. The tubes are removed from the water bath and allowed to stand for 30 min. The intensity of the colour is measured in a spectrophotometer at 540 nm setting the blank to zero OD.

A calibration curve is prepared with standard solutions containing 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 1.6 and 2.0 mg ascorbic acid/100 ml prepared in 6% TCA. OD readings are plotted against concentrations. Ascorbic acid concentration in the test solution is read from the standard curve.

The calibration curve is checked from time to time. This method has yielded recoveries ranging from 96 to 104% of added ascorbic acid in plasma samples.

Note :

Determination of ascorbic acid has to be carried out immediately after the collection of the sample.

(iii) Leucocytes

Reagents :

1. Diluent : 50 ml of 6% dextran solution, 200 ml physiological saline and 2.0 ml 10% sequesterine (sodium salt of ethylene diamine tetraacetate) are mixed well.

To 0.1 and 0.2 ml aliquots of the filtrate taken in test tubes, 0.5 ml of ethylmethylketone and 0.2 ml of 5 N NaOH are added mixed and kept at room temperature for 5 min. Add 0.3 ml of 5N HCl vortex and keep in a boiling water bath for 5 min. The tubes are cooled and 1 ml of KH_2PO_4, followed by 7 ml of water are added to make up the volume to 10 ml. Blanks are run with similar aliquots of the filtrate except 0.3 ml 5N HCl is added in the beginning, before adding the ketone. Standards containing 0.2 to 1.0 μg of NAD at 5 levels are run along with each set of determinations.

The fluorescence is read in a Farrand spectrofluorimeter at wavelengths, 365 nm and 450 nm for excitation and emission respectively.

The concentration of total nicotinamide nucleotides in the samples are calculated from the fluorescence of the NAD standards, simultaneously run along with the samples.

Note:

N^1-Methyl nicotinamide: Urinary N^1-methyl nicotinamide is estimated fluorimetrically. Half ml of the urine sample is taken each for blank, test and recovery. Two concentrations (0.1 and 0.2 μg) of standard N^1-methyl nicotinamide are added to the urine samples in the recovery tube. The total volume in each tube is made upto 1 ml.

The rest of the procedure adopted is the same as described for the estimation of nicotinamide nucleotides.

N^1-methyl nicotinamide content of each urine sample is calculated separately based on the recovery of standard N^1-methyl nicotinamide. (Recoveries ranged from 80–94%).

Reference:

J. Biol. Chem. **167**, 169 (1947).

h. Ascorbic acid

(i) Food

(ia) Dye method:

Principle:

The blue colour produced by the reduction of 2,6 – dichlorophenol indophenol by ascorbic acid is estimated colorimetrically.

Reagents:

1. Acetate buffer, pH 4.0: 300 g of anhydrous sodium acetate, 700 ml of water and 1 l of glacial acetic acid are mixed.

2. Dye solution: 25 mg of the sodium salt of 2,6 – dichlorophenol indophenol is dissolved in distilled water and made upto 200 ml.

3. 6% Metaphosphoric acid (HPO_3).

4. Ascorbic acid standard (1 mg per ml): 100 mg of pure ascorbic acid dissolved in 100 ml of 6% HPO_3

Procedure :

A weighed quantity of the material is blended with a convenient volume of 6% HPO_3 and the slurry diluted to obtain a final mixture containing approximately 20 μg of ascorbic acid per ml (for example, in case of leaves, 5 g of the sample can be blended with 6% HPO_3 to make 50 ml and 5 ml of the slurry further diluted to 50 ml).

The mixture is then filtered and 2 – 5 ml of the filtrate placed in a 50 ml seperating funnel (A). The same amount of the extractant (6% HPO_3) is taken in two more separating funnels, B and C. Funnel B serves as the dye blank. and to funnel C which serves as a standard is added 0.1 ml (equivalent to 0.1 mg ascorbic acid) of the ascorbic acid standard solution. An amount of acetate buffer equal to the volume of the extract taken is then added to all the three funnels, followed by 2 ml of the dye solution. Xylene 10 ml is then added quickly and the contents shaken for 6–10 sec. After the layers separate, the lower water layer is removed and the colour in the xylene extract measured in a photoelectric colorimeter at 500 nm.

Calculation :

The calculation for the ascorbic acid content is based on the reduction in the OD of the dye solution on reaction with ascorbic acid. If the ODs of the extracts from funnels A, B and C are a, b and c, then the ascorbic acid contained in the amount of the extract taken for reaction with the dye is equal to $\frac{0.1\ (b-a)}{b-c}$ mg. The ascorbic acid content of the material can then be calculated by applying the necessary dilution factors.

Note :

It has been found that certain batches of metaphosphoric acid have impurities that reduce the dye. Hence the reagents used should be checked to guard agaist this possibility. Besides a reagent blank with the addition of the dye should always be run along with the sample.

(ib) Dinitrophenylhydrazine method :

Principle :

This procedure is based on the oxidation of ascorbic acid to dehydroascorbic acid followed by coupling with 2, 4 – dinitrophenylhydrazine under controlled conditions to give red coloured osazones. A comparison of the colour produced in sample and ascorbic acid standard solutions, is used as a means of determining ascorbic acid contents of the foodstuff.

Reagents :

1. 9 N H_2SO_4
2. 2% 2,4 – Dinitrophenylhydrazine
3. 2% Thiourea in 5% metaphosphoric acid
4. 85% $H_2\ SO_4$
5. Ascorbic acid standard
6. 5 and 10% Metaphosphoric acid

Procedure :

Extraction : A weighed sample (about 10 g) estimated to contain 0.5–1.0 mg of ascorbic acid, is placed into a mortar and ground with 10 ml of 5% metaphosphoric acid and 1 g of dry stannous chloride.

2. 2, 4-Dinitrophenylhydrazine : 2.2 g of 2, 4-dinitrophenylhydrazine is dissolved in 10 N H_2SO_4. To this is added 5 ml of 5% thiourea solution followed by 5 ml of 0.6% $CuSO_4$. 5 H_2O and the solution filtered.

3. 5% TCA.

4. 65% H_2SO_4 : To 30 ml of distilled water is added 70 ml of concentrated sulphuric acid.

Procedure :

Separation of leucocytes : About 3 ml of freshly drawn whole blood is added to a siliconised tube containing 12.4 ml of dextran saline sequesterine solution, mixed and allowed to stand for $\frac{1}{2}$ hr at room temperature, during which time most of the erythrocytes sediment to the bottom. The supernatant fluid containing total leucocytes and platelets (hereafter leucocytes and platelets will be referred to collectively as leucocytes) is removed and thoroughly mixed.

A measured volume of fluid (usually containing 10-15 million leucocytes) separated as above containing total leucocytes is centrifuged at 3000 rpm for 15 min and the remaining portion of the fluid is used for cell count. Leucocyte counts are performed using a haemocytometer. The supernatant is drained by inversion. The cellular deposit is thoroughly homogenised by adding 1.3 ml of 5% TCA.

The tubes containing the homogenised deposit is centrifuged to remove cell debris. One ml of the resulting supenatant fluid is removed to a test tube, 0.3 ml of 2,4-dinitrophenylhydrazine reagent added and incubated at 37°C for 4 hr. After cooling in ice water bath, 1.5 ml of 65% H_2SO_4 is added and the OD read in spectrophotometer at 520 nm against a reagent blank.

A standard curve is prepared by using concentration 0.4, 0.8, 1.6, 4.0 and 6.0 μg of ascorbic acid/ml prepared in 5% TCA, these being treated identical to the test solutions. The readings of the test solutions are read off the straight line standard graph obtained. The ascorbic acid content of leucocytes is calculated as follows and the concentration is expressed as $\mu g/10^8$ leucocytes,

$$\mu g/10^8 \text{ leucocytes} = \frac{\text{Graph reading} \times 1.3 \times 10^8}{\text{No. of leucocytes in the aliquots used for estimation}}$$

This method of expression is adopted instead of the mg/100 g which is usually employed to obviate inaccuracies which are likely to occur, when small samples of leucocytes with adhered fluid are weighed.

References :

1. J. Biol. Chem. **147**, 399 (1943).
2. Clin. Sci. **21**, 157 (1961).
3. Lancet, **1**, 468 (1964).

i. Retinol

(i) Fluorimetric procedure

Protein bound vitamin A is released and vitamin A esters hydrolysed from serum by hot alcoholic potassium hydroxide. The vitamin A alcohol formed is extracted with xylene. The xylene layer is separated and excited at 350 nm and the fluorescence measured at 490 nm. Non-specific fluorescence, if any, is measured at the same wavelengths following UV destruction of vitamin A in the xylene extract and subtracted from the total fluorescence. to yield the net fluorescence due to vitamin A.

Reagent :

Alcoholic potassium hydroxide : Just before use dilute 10 N KOH to 1 N with 95% double distilled alcohol.

Procedure :

Serum 50 μ l is taken into a 3 ml glass centrifuge tube and an equal volume of freshly prepared 1N alcoholic KOH is added, and mixed in a vortex mixer. The tubes are then kept in a water bath, maintained at 60°C for 20 min. The tubes are cooled and 0.3 ml xylene is added and vitamin A is extracted by vigorously mixing the two phases in a micro vortex mixer for 20 sec. The upper xylene layer is separated by centrifuging and transferred to micro cuvettes. Fluorescence is measured in a Farrand fluorimeter at 490 nm with excitation at 350 nm. The cuvettes are then covered with glass beads to avoid evaporation and irradiated for 10 min in an irradiation chamber. Fluorescence reading are again taken following irradiation and the net fluorescence destoryed by UV irradiation is calculated.

A series of standards of vitamin A palmitate containing 0.01 to 0.65 μg in 50 μl are treated in a similar manner as that for the serum samples. Simultaneously, the absorbance of an alcoholic solution of the standard containing approximately 3 μg/ml of vitamin A plamitate is measured in a Beckman DU spectrophotometer at 328 nm.

Calculation :

The exact vitamin A content of the standard from the absorption reading is calculated using the conversion factor $E^{1\%}_{1\ cm} = 940$. A standard curve is constructed and the vitamin A content of the sample is estimated from this.

References :

1. J. Biol. Chem. **166**, 177 (1946).
2. J. Am. Chem. Soc. **64**, 2407 (1942).
3. Clin. Chem. Acta. **27**, 165 (1970).

(ii) Spectrophotometric procedure

Principle :

The absorption at 460 nm and the difference in absorption at 326 nm before and after irradiation with ultraviolet light (between wavelengths 310–400 nm) can be used as a measure of carotene and vitamin A respectively.

Reagents :

25% Alcoholic KOH : 1 N KOH in 90% ethanol is prepared fresh and used for saponification.

Procedure :

Extraction and saponification : In a glass stoppered tube 3–4 ml of serum and an equal volume of alcoholic KOH are taken and mixed well. The tube is incubated in a water bath at 60–65°C for 20 min, removed and allowed to cool to room temperature. The unsaponifiable matter is then extracted three times with 10 ml of light petroleum ether (after the first extraction, the aqueous phase should be diluted further with water to ensure complete extraction). The petroleum ether extract is washed with water to remove

ıe alkali, passed through anhydrous sodium sulfate and evaporated to dryness under vacuum at 40°C. ıe dry residue is immediately dissolved in 3-4 ml cyclohexane (same volume as serum).

The OD of the cyclohexane soultion is measured at 328 nm and at 460 nm in a spectrophotometer. ıe solution is now transferred to a soft glass tube with a stopper and irradiated with UV light (The mp should be turned on 10 min before use). The tube should be kept at a distance of 20 cm from the mp. The OD at 328 nm is again read and the difference in OD is taken as a measure of vitamin A in e solution.

Calculation :

$E^{1\%}_{1cm}$ 328 of vitamin A in cyclohexane = 1550, and $E^{1\%}_{1cm}$ 460 for β-carotene = 2100.

μg of vitamin A/100 ml serum = (E 328 initial – E 328 irradiated) x 645.

μg β – carotene in 100 ml serum = E 460 x 477.

Note :

This method can be scaled down to one ml serum also, provided micro cuvette for the spectrophotometer are used to read a small volume of cyclohexane extract.

References :

1. J. Biol. Chem. **166**, 177 (1946).
2. Am. J. Clin. Nutr. **8**, 833 (1960).

(iii) Carr-Price method

Principle :

Vitamin A gives a blue coloured complex with antimony trichloride which has an absorption aximum at 620 nm. The intensity of this bule colour can be taken as a measure of vitamin A. Since -carotene also gives a coloured complex with antimony trichloride, a correction has to be employed.

Reagent :

25% Antimony trichloride: 25% solution (W/V) of the reagent in chloroform containing 1% /V) acetic anhydride.

Procedure :

An aliquot of the vitamin A solution in chloroform is taken in the spectrophotometer cuvette and the lume made upto 1 ml. The slit width of the instrument is adjusted at 620 nm with chloroform as blank. vo ml of antimony trichloride solution is added and the intensity of the blue colour read at 620 nm thin 15 sec.

Standard curves are plotted with different concentrations of pure vitemin A and β-carotene. Cor- tion for β-carotene. can be made by estimating the actual amount of β-carotene in the solution from e OD at 460 nm as described earlier. The OD for that amount of carotene at 620 nm in the Carr- ce test is obtained from the standard curve and that value subtracted from the total OD at 620 nm D 620 nm observed-OD 620 nm β-carotene = OD 620 nm corrected for vitamin A in the serum).

(iv) HPLC procedure :

Principle :

The vitamin A is extracted with a suitable organic solvent and an aliquot of the organic phase is

injected onto a normal or reversed phase HPLC column, followed by an eluting solvent of suitable polarity. Retinol, which is eluted as a sharp peak within 1–6 min is detected by a sensitive UV detector set at 325–328 nm. Retinol is quantitated by use of peak height ratios or peak area ratios relative to an internal standard (retinyl acetate or other appropriate analogs).

Reagents :

All organic solvents should be checked for absorbancy at the detection wavelength and purified by distillation or by passage through a silicic acid column before use. Solvents used for HPLC must be degassed and free of particles.

Standards : A stock standard solution of retinyl acetate in ethanol (50 μg retinol/ml) is prepared by dissolving about 1 mg of retinyl acetate in 10 ml of ethanol, determining the concentration in a 1/30 dilution of an aliquot in ethanol by use of the $E^{1\%}_{1cm}$ at 325 nm as 1795, and then diluting the stock standard appropriately with ethanol. A sample calculation follows :

A 1 : 30 dilution of a solution of all-trans retinyl acetate, prepared and assayed as indicated above, gave an absorbancy reading at 328 nm of 0.58 in ethanol. The concentration of vitamin A expressed as retinol in this solution is :

$$\frac{0.58 \times 30}{0.1795} \text{ or } 96.9\,\mu \text{ g/ml}$$

The 10 ml of this solution is then diluted to 19.4 ml to yield the stock standard with 50 μg retinol/ml.

Procedures :

1. Transfer 100 μ l of serum (or plasma), 15 μl of internal standard solution (4 μg/ml of retinyl acetate or propionate) and 100 μl of methanol to a conical centrifuge tube. Mix the contents of the tube with a vortex mixer. Add 200 μ l of extraction solvent (petroleum ether 80, dichloromethane 19.3, isopropanol 0.7, by volume) and cap the tube. Extract bv interrupted mixing on the vortex mixer for 60 sec. After centrifugation (3000 rpm, 2 min) inject 100 μ l of the supernate on top of the column by use of a Hamilton syringe. Elute with the same solvent as used for extraction.

2. Pipette 100 μ l of plasma (or serum) and 100 μ l of the internal standard solution in ethanol (0.4 - 0.6 μg of retinyl acetate/ml) into a glass test tube. Mix the contents vigorously on a vortex mixer for 10-20 sec. Add 120 μl spectrograde hexane and mix the contents vigorously but intermittently for 45 sec on a vortex mixer, making sure that the bottom layer is thorougly extracted. After centrifugation (2200 rpm, 5 min) to ensure phase separation, carefully transfer 75 μ l of the upper hexane layer to a 3 or 5 ml conical centrifuge tube. If some of the lower phase is drawn into the pipette, abnormal results will be obtained. Evaporate the solvent, preferably under a stream of nitrogen, in a 60°C water bath. Be sure that water vapour does not condense on the inside of the tube during evaporation or cooling. Immediately dissolve the lipid residue in 25 μl diethyl ether followed by 75 μl methanol. Gently swirl and tap to enhance solubility of the lipid residue. Inject about 90 μl of the solution on the column with a 100 μ l syringe. Elute with filtered methanol : water (95 : 5).

Chromatographic conditions :

	Procedure 1	Procedure 2
Column	15 x 0.2 cm i.d. Micro Pak Si-10	30 x 0.39 cm i. d. Micro Bonda-Pak C-18
	Petroleum ether/dichloromethane/ isopropanol (80/19.3/0.7 by vol)	methanol/ water (95/5 by vol)

	Procedure 1	Procedure 2
Flow rate	0.5 ml/min	2.5 ml/min
Pressure	10 Kg/cm^2	Not specified
Detector wavelength	328 nm	328 nm for retinol; 280 nm for retinol and alphatocopherol
Detection sensitivity	0.04 AUFS on recorder	0.01 AUFS on recorder
Temperature	Ambient	Ambient
Recorder	Not specified	10 mV, 1 cm/min
Elution time :		
Retinol	5.0 min	2.2 min
Internal standard	6.2 min	3.0 min

Calculation :

By use of an internal standard, losses due to incomplete extraction, inaccurate aliquots, oxidation, etc , are automatically corrected, provided that the internal standard has physical and chemical properties sufficiently similar to retinol, is suitably separated from retinol on HPLC, does not coincide with other 325 nm absorbing materials in serum, and is not converted to retinol under the assay conditions. A precisely known amount of the internal standard is added to the aliquot of plasma to be analysed. By determining the relative extraction efficiency and detector response of retinol and the internal standard, a standard curve is fashioned in which the ratio of peak heights (or areas) is plotted against the retinol concentration in plasma. In experimental samples, the peak height (or area) ratio is determined and the appropriate plasma retinol concentration determined from the standard curve or regression line formula.

A standard curve is prepared by adding varying amounts of retinol (i.e. 10, 20, 40, 80 and 120 ng) to a fixed amount (i.e. 50 ng) of internal standard in a final volume of 100 μ l of eluting solvent, injecting the solution on HPLC under assay conditions, measuring the peak heights, and calculating the peak height ratio. The peak height ratio is then plotted as the abscissa with the plasma retinol concentration (for a 100 μ l plasma aliquot) as the ordinate.

Note :

The stock standard solution is stable for more than a week when kept at 4°C in the dark, but should be checked periodically for deterioration both by HPLC and by spectra. A working standard is prepared atleast weekly. Plasma samples kept in the frozen state at – 20°C give the same values for atleast 2 months.

References :

1. Clin. Chem. **22**, 1593 (1976).
2. Am. J. Clin. Nutr. **32**, 2143 (1979).
3. Vision Res. **20**, 355 (1980).
4. J. Chromatogr. **168**, 125 (1979).

j. β - Carotene

Principle :

The individual carotenoids are separated on a column of calcium hydroxide or alumina and determined spectrophotometrically. The values for their respective vitamin A potency are used to arrive at the total vitamin A value of the foodstuff.

Procedure :

Preparation of the sample : Twenty five g of freshly ground sample is allowed to stand overnight in a mixture of 100 ml of petroleum ether-acetone (1 : 1). The extract is then filtered and the residue on the filter, washed twice with successive 500 ml portions of the mixture till all the yellow colour is extracted. The pooled filtrate is shaken with 50 ml portions of water and water washings discarded. This is repeated twice. The solvent layer is then dried over anhydrous sodium sulphate and concentrated under reduced pressure to a final volume of 4 ml.

Separation of pigments using calcium hydroxide column : A column of 30 x 1 cm is packed with 6 g of calcium hydroxide under gentle suction. About 1 – 2 g of anhydrous sodium sulphate is placed on top of the packed column. The column is initially wet by passing petroleum ether.

Two ml of the concentrated extract containing about 40-100 μg of the total carotene is charged on to the column. Light suction is employed and a 1% acetone in petroleum ether is used as developing solvent.

The separated bands are individually eluted and their spectra studied in a Beckman DU spectrophotometer to identify the different carotene fractions.

For quantitative estimation of different carotene fractions, standard graphs are obtained with graded concentrations of pure β-carotene dissolved in petroleum ether and this curve is used for determining the concentrations of the solutions of pigments whose main absorption is around 460 nm.

Separation using alumina column : Two ml of the concentrated carotene extract is loaded on to a column of alumina (10 x 1 cm) containing 3% anhydrous sodium sulphate and eluted with petroleum ether. The β – carotene is now eluted with petroleum ether containing 3% acetone. The volume of this eluate is made upto 5 ml and OD of this eluate is measured at 450 nm in Beckman DU spectrophotometer.

Calculation :

1 OD is equivalent to 4 μg/ml of β-carotene when measured in 1 cm cell.

The relative potencies of different carotenoids reported in the literature are used to calculate the vitamin A value in the case of each carotenoid fraction. The sum of the values of all the fractions present in the chromatogram of a particular foodstuff gives its true vitamin A value.

Reference :

Ind. J. Nutr. and Dietet. 4, 10 (1967).

k. 25 - Hydroxy vitamin D_3

Principle :

The principle involved in this method is that of competitive protein binding assay.

Reagents :

1. ^{3}H (26,27) – 25 OHD_3 (80 Ci/m mole) : This is supplied in toluene. Take an appropriate aliquot and dry under nitrogen. Redissolve in absolute ethanol.

2. Standard 25-OHD_3 (stock standard-16 μg/ml) : Weigh exactly 175 μg 25-OHD_3 in a screw capped test tube. Add 10.94 ml 95% ethanol and dissolve. Flush with nitrogen and store at - 20°C.

Working standards (A): Take 0.1 ml stock standard and make up the volume to 10 ml with 95% ethanol (16 ng/0.1 ml). Make further dilutions as follows:

	Standard (ml)	Ethanol (ml)	Final concentration (ng/50 μ l)
B	0.5 A	0.5	4.0
C	0.3 A+0.3 B	—	6.0
D	0.3 C	0.3	3.0
E	0.3 B	0.3	2.0
F	0.3 E	0.3	1.0
G	0.3 F	0.3	0.5
H	0.3 G	0.3	0.25
I	0.1 G	0.4	0.10

3. Phosphate buffer: 0.05 M sodium phosphate buffer stock solution is prepared from 13.6 g sodium phosphate (mono basic) dissolved in 1.5 l of water. 0.4 N NaOH is added to get a pH of 7.5 and the solution made upto 2 l with water.

4. Binding protein: Draw blood from a normal albino rat and separate the serum. Store aliquots of 0.1 ml at – 20°C. On the day of use dilute 0.1 ml to 100 ml with assay buffer.

5. Charcoal – Dextran suspension: Suspend 5 g activated charcoal (Norit A) and 0.5 g dextran T–80 in 100 ml phosphate buffer.

6. Bray's scintillation fluid: (Refer Appendix)

Procedure:

Serum extracts: Take 0.5 ml serum in a stoppered test tube and add 2 ml 95% ethanol. Mix on a vortex shaker for 1 min. Let stand at 4°C for 10 min. Centrifuge at 2500 rpm for 10 min at 4°C. Decant the alcohol layer and store.

Take standard or serum extract (50 μ l) and add 20 μ l of ^{3}H 25-OHD_3 and 500 μ l of binding protein. Mix, incubate at 4°C for 24 hr. Then add 100 μ l of charcoal dextran suspension, mix for 30 sec and incubate at 4°C for 30 min.

Centrifuge at 3000 rpm for 30 min at 4°C. Pipette out carefully 0.5 ml of the supernatant and add to 10 ml scintillation fluid. Count radioactivity in a liquid scintillation counter.

Run appropriate blanks for each tube, by eliminating binding protein. Bound radioactivity in blank tube is nonspecific binding.

Calculate per cent radioactivity bound, after subtracting nonspecific binding. Plot the values against concentration of 25–OH D_3 and read values of unknown off the graph.

References:

1. J. Clin. Endocrinol. Metab. **33**, 554 (1971).
2. J. Clin. Endocrinol. Metab. **33**, 992 (1971).
3. Clin. Chim. Acta. **72**, 383 (1976).

I. Vitamin E

(i) TLC

Principle:

Vitamin E is extracted from plasma after saponification. After removal of the interfering substances,

vitamin E is estimated with a $FeCl_3$ – bathophenanthroline – H_3PO_4 reagent system. Vitamin E reduces ferric ion to ferrous ion quantitatively, which then combines with bathophenanthroline to from an orange coloured complex. After adding H_3PO_4 to stabilise the complex, the colour is read at 534 nm.

Reagents :

1. Saturated KOH
2. Pyrogallol : Dissolve 200 mg of pyrogallol in 10 ml absolute alcohol (freshly prepared).
3. $FeCl_3$: 0.005M in alcohol
4. Bathophenanthroline : 0.2% in alcohol
5. Orthophosphoric acid : 0.001M in alcohol.
6. Methanolic rhodamine 6G, 0.001% : Make a stock solution of 100 mg in 100 ml of methanol. To prepare a working solution, pipette out 1 ml of the stock and make upto 100 ml.

Procedure :

Saponification : Pipette out 1 ml of plasma into a stoppered 15 ml test tube. Add 2 ml of 2% solution of pyrogallol with mixing. Keep the solution at 70°C in a waterbath for 2 min, after which 0.3 ml of saturated KOH is added. After thorough mixing, keep the tube at 70°C for 30 min. Cool the tubes. Add 1 ml of water followed by 4 ml of hexane. Shake the contents vigorously for 5 min. Centrifuge the solution to separate the phases. (The upper phase contains the hexane extract of vitamin E).

TLC : Pipette out 3 ml of hexane extract into another centrifuge tube and evaporate it under nitrogen. Dissolve the evaporated material in 25-50 μl of chloroform. Spot it on a silica gel G plate, activated before use. Standard tocopherol (vitamin E) is also spotted along side for reference. Develop the spot in a solvent system of benzene : ethanol (99 : 1). After development, spray the standard tocopherol spot only with rhodamine 6 G, covering the test spots, with a glass plate. The spot becomes visible on examining it under UV light. Scrape the corresponding area from the test spots into 7 ml centrifuge tube. Add 1.5 ml of absolute alcohol. Mix the contents thoroughly in a vortex mixer. Centrifuge the contents at 2500 rpm for 5 min.

Estimation : One ml of the above ethanol extract is taken for estimation. Add 0.2 ml 0.2% bathophenanthroline. Mix the contents thoroughly. (Assay proceeds rapidly from this point, solutions are not exposed to direct light). Add 0.2 ml of ferric chloride reagent followed by vortex mixing. After 1 min, add 0.2 ml phosphoric acid reagent. Mix the contents thoroughly. Determine the absorbance of this solution at 534 nm in a Beckman spectrophotometer.

Standards : A stock solution of 100 μ g/ml of pure DL-tocopherol is prepared; suitable dilutions are made to obtain concentrations of 2, 4, 6, 8 and 10 μ g. All standards are uniformly made upto 1 ml with absolute alcohol and assayed.

Construct the standard graph from which the sample concentration is read. Express the values as μ g/100 ml.

Note :

Since vitamin E is photosensitive, the assay should be carried out in subdued lighting.

Recovery experiment is always carried out by adding known quantity of vitamin E serum and calculating the recovery percentage. This factor is taken into consideration during calculation of the values.

Reference :

J. Lipid Res. **14**, 533 (1973).

(ii) Column chromatography

Principle :

This procedure is adopted for samples which have high fat content such as milk. The high fat content in milk interferes during the TLC separation of vitamin E and hence this procedure is adopted.

Reagents :

Same as in TLC procedure.

Procedure :

Take 1 ml of milk or plasma, add 2 ml of 2% alcoholic pyrogallol and mix. Keep in the water bath at 70°C for 2 min. Add 0.3 ml of saturated KOH, mix well, saponify for 30 min and cool. Add 1 ml of water followed by 4 ml of hexane and shake it vigorously. Centrifuge and remove the top hexane layer as much as possible into another test tube. Add equal volume of water, mix them well and centrifuge. Take the top hexane layer containing vitamin E. Repeat the washing procedure once more, after which 3 ml of the hexane extract is taken out and placed in a test tube. Evaporate it under N_2. Add exactly 5 ml of reagent grade hexane to the residue.

Weigh out 2.5 g of activated neutral alumina (E. Merck) and make it up in a glass column with 2% alcohol in hexane. Stir and remove the air bubbles. Run off the solvent. When the solvent is just above the alumina layer, add 5 ml of hexane and run it off. Now add 1 ml of 1% acetone in hexane followed by 5 ml of the above hexane extract. Collect fractions of 2 ml each (tubes 1–3). Follow it up with another 10 ml of 1 % acetone in hexane. Collect 5 fractions of 2 ml each (tubes 4–8). (This step removes the interfering substances such as β-carotene). Add 2 ml of 20% alcohol in hexane, collect it in 2 tubes as one ml eluate fractions (tubes 9-10). Add 8 5 ml of the same eluant again. Collect as 1 ml fractions (tubes 11-16). Pool the fractions in tubes 9–14, which contain vitamin E, evaporate under nitrogen. Make up the residue to 2 ml with absolute alcohol. Take 1 ml for estimation.

The spectrophotometric estimation of vitamin E is carried out as detailed earlier in the previous procedure.

Note :

The solvents used are all double distilled. Neutral alumina is activated at 100°C for 1–2 hr just prior to use. Recovery experiment as detailed in the previous procedure is carried out and the final values corrected for 100% recovery.

Reference :

Nutr. Rep. Int. **23**, 135 (1981).

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VII. MINERALS

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1. PROCESSING OF SAMPLE

a. Ash solution :

The ash (refer food analysis) is moistened with a small amount of glass distilled water (0.5 1.0 ml) and 5 ml of distilled hydrochloric acid is added to it. The mixture is evaporated to dryness on a boiling water bath. Another 5ml of hydrochloric acid is added again and the solution evaporated to dryness as before. Four ml of hydrochloric acid and a few ml of water are then added and the solution warmed over a boiling water bath and filtered into a 100 ml volumetric flask using Whatman No 40 filter paper. After cooling, the volume is made upto 100 ml and suitable aliquots are used for the estimation of phosphorus, iron and calcium.

b. Direct solution :

Some liquid samples can be analysed directly without pretreatment and after dilution with water or reagent. Serum is diluted 5 times with 8% butanol in deionised water for mineral determination. The butanol is checked for its mineral contamination. Serum equivalent solution is prepared by 7. 6 g NaCl and 0. 29 g KCl in 100 ml deionised water and is used as a blank.

Fruit juices and beverages such as tea, fresh fat-free milk (freshly collected milk centrifuged for 5-10 min and kept in ice for 5 min and the upper fat layer is removed) and wine can be analysed after proper dilution in a flame or carbon rod analyser.

c. Wet digestion :

Organic matter in food materials or solids can be destroyed and oxidised by boiling with sulphuric, perchloric and nitric acids.

1. Weigh about 3 g of dry ground food sample in a 100 ml Kjeldahl flask.
2. Add 25 ml 3 : 2:1 (conc. nitric acid-conc. perchloric acid-conc. sulphuric acid) mixture and shake well ensuring that no dry food lumps remain. Add a clean glass bead (acid washed).
3. Leave aside for 3-4 hr or preferably overnight in a fume cupboard. (Watch for foaming during the first hour especially with samples high in fat. If foaming occurs and threatens to overflow immerse the bulb of the flask in cool water till froth subsides).
4. Heat for about 30 min cautiously until the initial vigorous reaction has subsided (dense yellow fumes will be evolved).
5. Heat more strongly for 4 hr until most of the nitrous fumes are removed.
6. Continue heating until white fumes of perchloric acid are evolved. If charring occurs or flask contents tend to become dry, remove from heat, cool and add 5ml of nitric acid and continue heating.
7. Carry a blank with each set of samples.
8. When cool, transfer quantitatively with 3-4 washings of deionised water the contents of the digestion flask to 15 ml graduated test tube. Make upto the 10 ml mark with deionised water and mix thoroughly. Allow the heated solution to cool and make up to the 10 ml mark again if necessary.
9. The tubes are centrifuged for 30 min.
10. Transfer the ash solution to acid-washed polythene bottles and store in a cool place prior to elemental analysis by atomic absorption spectrophotometry.

Note :

Use only glass distilled or deionised water. Nitric acid diluted 1 to 100 in deionised water is used as glassware washing liquid. All operations should be carried out in a fume cupboard with proper ventilation.

2 ATOMIC ABSORPTION SPECTROSCOPIC DETERMINATION OF MINERALS

Representative sample in a suitable liquid form is sprayed into the flame of an atomic absorption spectrophotometer and the absorption or emission of the mineral to be analysed is measured at a specific wavelength.

Reagents :

Standard solutions :

1. Stock solutions, 1000 μg/ml : Dissolve 1g mineral equivalent of mineral salt in a minimum amount of redistilled hydrochloric acid (about 10 ml). Dilute to 1 l with deionised water.

2. Working standard solutions : Dilute aliquots of the stock solutions with deionised water to make atleast 4 standard solutions of each element within the range of determinations (Refer table).

Procedure :

1. Depending upon the mineral to be determined standards and blank solutions may be aspirated into the flame directly or after suitable dilutions to attain working range of the instrument.
2. The optimum operating conditions recommended by the instrument manufacturer should be used.
3. Read atleast 3-4 ranges of standard solutions before and after sample readings. Flush burner with deionised water between samples and check for zero setting.
4. Prepare calibration curve from the readings of standards.
5. Determine the concentration of samples from the standard graph.

Calculation :

$$\text{ppm mineral} = (\mu \text{ g mineral/ml}) \times \frac{\text{dilution factor}}{\text{ml aliquots x g sample}}.$$

OPERATION PARAMETERS (Varian Techtron AA – 6)

Mineral	Wavelength nm	Flame	Optimum working range, μ g/ml
Ca (dilute with 0.1% lanthanum chloride solution)	422.7	Rich air – C_2H_2 (Oxidising)	0.5 – 2.5
Cr	357.9	Rich air – C_2H_2 (reducing)	2.5 – 10.0
Cu	324.7	Air – C_2H_2 (oxidising)	2.0 – 8.0
Fe	248.3	Rich air – C_2H_2 (oxidising)	2.0 – 8.0
Mg	285.2	Rich air – C_2H_2 oxidising)	0.1 – 0.6
Mn	279.5	Air – C_2H_2 (oxidising)	1.0 – 4.0
Zn	213.9	Air – C_2H_2 (oxidising)	0.4 – 1.6

3. CALCIUM

a. Titrimetric method

Principle :

Calcium is precipitated as oxalate and is titrated with standard potassium permanganate.

Reagents :

1. 4% ammonium oxalate solution
2. Dilute ammonia solution (2 ml of liquor ammonia + 98 ml water)
3. 1 N H_2SO_4
4. 0.01 N Potassium permanganate solution
5. 0.01 N Oxalic acid : Sodium oxalate is dried in an oven at 100 – 105°C for 12 hr. Exactly 0.67 g is dissolved in redistilled water, 5 ml concentrated H_2SO_4 is added and solution made upto 1 l after it has cooled down.

Standardisation of potassium permanganate solution : 25 ml of 0.01 N oxalic acid is transferred

to an Erlenmeyer flask. One ml of concentrated H_2SO_4 is added, warmed to about 70°C and titrated against $KMnO_4$ solution, till the faint pink colour remains.

Procedure:

Two ml of sample is taken into a 15 ml centrifuge tube. Add 2 ml of distilled water and 1 ml of 4% ammonium oxalate solution and mix thoroughly and leave overnight. Again the contents are mixed and centrifuged for 5 min at 1500 rpm. The supernatant liquid is poured off and the centrifuge tube drained by inverting the tube for 5 min on a rack (care should be taken not to disturb the precipitate). The mouth of the centrifuge tube is wiped with a piece of filter paper. The precipitate is stirred and the sides of the tubes are washed with 3 ml of dilute ammonia. It is centrifuged again and drained as before. The precipitate is washed once more with dilute ammonia to ensure the complete removal of ammonium oxalate. The precipitate is dissolved in 2 ml of 1 N H_2SO_4. The tube is heated by placing it in a boiling water bath for 1 min and titrated against 0.01 N $KMnO_4$ solution to a definite pink colour persisting for atleast 1 min.

Calculation :

1 ml of 0.01 N $KMnO_4$ is equivalent to 0.2004 mg of calcium.

$$\text{mg of calcium/100 ml serum} = (X-b) \times 0.2004 \times \frac{100}{2} \text{ where,}$$

X = number of ml of 0.01 N $KMnO_4$ required to titrate the sample,

b = number of ml of 0.01 N $KMnO_4$ required to titrate 2 ml of H_2SO_4 (blank).

If the normality of $KMnO_4$ is N, the value obtained in the above formula should be multiplied by the factor, $\frac{N}{0.01}$.

Note :

Potassium permanganate solution needs to be frequently standardised.

Reference :

Practical Physiological Chemistry, Hawk, Oser and Summerson (Eds.) 13th Edn., p. 644 (1957).

b. Colorimetric method

Principle :

Calcium forms a colour complex with the O-cresolphthalein dye, which is made more specific in the presence of 8 quinolinol.

Reagents:

1. Standard $CaCl_2$: 10 μ g Ca^{++}/ml prepared by dissolving required amount of anhydrous $CaCO_3$ in dilute HCl and making upto a definite volume with water.

2. Ammonia/ammonium chloride buffer, pH 10.5 : Dissolve 0.24 g NH_4Cl in 100 ml of 5% NH_4OH solution (V/V).

3. O-Cresolphthalein solution, 0.1%: 100 mg of O-cresolphthalein dissolved in 28 ml of ammonia/ammonium chloride buffer and diluted to 100 ml with water.

4. 8 Quinolinol: 1 % solution in absolute ethanol.

Procedure:

Different aliquots of the $CaCl_2$ solution with Ca^{2+} content varying from 2–10 μ g are taken in graduated stoppered tubes and volume in each case is made upto 5.5 ml with water. Subsequently, 1 ml of 8-quinolinol solution and 2.5 ml ammonia/ammonium chloride buffer are added and the volume adjusted to 9.0 ml. The contents are finally treated with 1 ml of O-cresolphthalein solution. After addition of each reagent mentioned above, the contents are thoroughly mixed. Water blank in the place of $CaCl_2$ solution is simultaneously carried throughout the procedure. Standards are read against blank in Spectronic-20 at 565 nm. Take readings after 5 min (stable upto 3 hr).

Reference:

Anal. Biochem. **20, 155 (1967).**

4. COPPER

a. Food

Principle:

Copper froms a coloured complex with sodium diethyl dithiocarbamate which is extracted and estimated colorimetrically.

Reagents:

1. EDTA solution: EDTA sodium salt 25 g is dissolved in 500 ml of glass distilled water. It is well shaken (4 to 5 times) with solid diethyl dithiocarbamate (0.5 g) and 100 ml of chloroform till the colour disappears. Eight ml of 0.1% cresol red is added and the mixture shaken well and the EDTA solution kept in a stoppered bottle.

2. Copper standard: Dissolve 982 mg of $CuSO_4$. 5 H_2O in water and 5 ml of concentrated H_2SO_4 is added and made upto 250 ml to give a standard solution of copper, 1 mg/ml.

3. 1% Sodium diethyl dithiocarbamate.

Procedure:

The dry materiel (2 g) is digested in a pyrex Kjeldahl flask with 5 ml of glass distilled water and 4 ml of conc. H_2SO_4 and 5 ml of distilled nitric acid. The flask is covered with a glass stopper throughout the digestion which is carried out on a burner with constant addition of nitric acid till a clear digest is obtained. Perchloric acid (1 ml) is then added and the digestion continued for 15–20 min. The digest is then quantitatively transferred to a separating funnel using glass distilled water to make to approximately 50 ml. Five ml of EDTA solution is then added and the solution neutralised with distilled ammonia (to get a pink colour). It is cooled and 1 ml of 1% sodium diethyl dithiocarbamate is added followed by 10 ml of distilled carbontetrachloride. The mixture is shaken for exactly 2 min. After the carbontetrachloride layer separates, it is run into colorimetric tube through a plug of cotton and the intensity of the colour read at 440 nm against a reagent blank extracted similarly.

For each set of estimations, a reagent blank prepared by using the same quantity of acids etc. is carried throughout the procedure. Copper in the same is calculated from a standard curve (0 – 40 μ g by applying the same method).

b. Blood

Principle :

Copper in serum or plasma reacts with sodium diethyl dithiocarbamate to form a coloured compound which can be measured spectrophotometrically.

Reagents :

1. 2 N HCl
2. 20% TCA (W/V)
3. Saturated solution of sodium pyrophosphate (fresh)
4. Saturated solution of sodium citrate (fresh)
5. Ammonium hydroxide diluted 2 : 1 with water (21 % NH_3)
6. 0. 1 % Solution of sodium diethyl dithiocarbamate
7. Standard copper solution containing 100 μg/ml: The standard is prepared by dissolving 392. 8 mg of $CuSO_4$. $5H_2O$ in glass distilled water and diluting the solution to 1 l. Only crystals which do not show signs of efflorescence should be used. Working standards are prepared from the stock solution to contain 0. 8 μg and 2. 0 μg per ml.

Procedure :

One ml of serum or plasma is taken and to it 1 ml of 2 N HCl is added and allowed to stand for 10 min followed by the addition of 1 ml of 20% TCA. After stirring it is kept for 10 min and then centrifuged for 30 min at 3000 rpm.

A 2.4 ml aliquot is collected and to it 0.2 ml each of sodium pyrophosphate and sodium citrate are added, followed by the addition of 0.4 ml of ammonium hydroxide. Glass distilled water is added to make up the volume to 3.3 ml.

The OD is read against a reagent blank containing no carbamate in Beckman DU spectrophotometer at 440 nm. After the OD is read, 0.2 ml of sodium diethyl dithiocarbamate solution is added to each tube including the reagent blank.

After mixing and keeping for 15 min the OD of the sample and blank are again read against the reagent blank containing no carbamate.

Calculation :

$$\text{Plasma or serum copper } (\mu g/100 \text{ ml}) = \frac{(D_2 - D_1)\ F \times K \times 3.0 \times 100}{2.4}$$

where,

D_2 = OD after adding the carbamate
D_1 = OD before adding carbamate
F = ratio between volume of the solution in the test tube before and after adding the carbamate
K = constant to be determined from the standard graph (concentration of copper/OD).

Note :

All glassware used should be thoroughly clean, acid washed and finally washed several times in glass distilled water.

Reference :

J. Biol. Chem.**196**, 209 (1952).

5. PHOSPHORUS

a. Fiske and Subba Row method

Principle :

In the determination of acid-soluble, lipid and total phosphorus, organic matter is destroyed by digestion with H_2SO_4 (or fuming nitric acid). The phosphate containing solutions are treated with molybdic acid to produce phosphomolybdic acid. This is reduced by the addition of 1, 2, 4-amino-naphtholsulphonic acid reagent giving a blue colour, the intensity of which is proportional to the amount of phosphate present.

Reagents :

1. 10% TCA solution in water
2. 10 and 5N H_2SO_4
3. 30 % H_2O_2 or fuming nitric acid
4. Aminonaphtholsulphonic acid reagent : 195 ml of 15% sodium bisulphite solution is taken in a glass stoppered cylinder and 0.5 g of 1, 2, 4-aminonaphtholsulphonic acid is added to it followed by 5 ml of 20 % sodium sulphite. It is stoppered and shaken until the powder is dissolved. If the solution is not complete, more of sodium sulphite, 1 ml at a time is added with shaking. The solution is then transferred to a brown glass bottle and stored in the cold. This solution is usable for four weeks.
5. Standard phosphate : Exactly 351 mg of pure dry monopotassium phosphate (KH_2PO_4) is dissolved in water and transferred quantitatively to a 1 l volumetric flask. Ten ml of 10 N H_2SO_4 is added and the solution diluted to the mark with water and mixed. This solution contains 0.4 mg of phosphorus in 5 ml. It is stable indefinitely.
6. 2.5% Ammoniummolybdate : 2.5 g of reagent grade ammoniummolybdate is dissolved in water, transferred to 100 ml volumetric flask, filled to the mark and mixed.

Procedure :

Preparation of TCA filtrate of whole blood : To 8 ml of a 10 % TCA solution, 2 ml of whole blood, serum or plasma is added slowly, with mixing. The mixture is shaken and filtered through a low ash filter paper.

Two ml of TCA filtrate of serum (equivalent to 0. 4 ml serum) is transferred to a test tube or micro-Kjeldahl flask and 2. 5 ml of 5 N H_2SO_4 is added; also add a quartz chip to minimise bumping. The contents of the flask are digesbed on a micro burner. After evaporation is complete and the mixture turns brown or black with no further change, the tube is removed, cooled slightly and 1 drop of 30 % H_2O_2 (if it gives too high a blank then 1 or 2 drops of fuming nitric acid) added to fall directly into the digestion mixture. The heating is then continued until the contents of the tube become colourless. When the mixture is clear, the tube is cooled and a few ml water is added and the solution is heated to boiling momentarily. The contents are then transferred to a 25 ml volumetric flask quantitatively with washings. To the flask is added 2. 5 ml of 2. 5 % ammoniummolybdate solution, followed by 1 ml of

minonaphtholsulphonic acid reagent. The solution is made upto 25 ml with water and mixed well. It is allowed to stand for 5 min, then the intensity of the colour is read in a colorimeter. For photometric measurements, the most accurate procedure is to run a digested blank and series of digested standard solutions along with each series of unknowns. The photometer is set to zero OD with blank and the colour of standard and unknown solutions are determined at 660 nm. The standard curve is plotted and the unknown read off the curve.

Calculation:

$$\text{mg of total acid soluble phosphorus per 100 ml serum} = \text{mg P in unknown} \times \frac{100}{0\ 4}.$$

Reference:

Hawk's Physiological Chemistry, Oser and Summerson (Eds), 14th Edn, p. 1115 (1965).

b. Micromethod

Principle:

Phosphate reacts with ammoniummolybdate to give rise to phosphomolybdate complex which is reduced to a more stable colour complex with ascorbic acid.

Reagents:

1. 10% TCA : Before using check the phosphorus content.
2. 10% Ascorbic acid : Dissolve 10 g of ascorbic acid in distilled water and dilute to 100 ml. Store under refrigeration at 2 – 4°C. The solution is stable for about 7 weeks.
3. 2. 5% Ammoniummolybdate : Dissolve 2. 5 g of ammoniummolybdate in distilled water and dilute to 100 ml.
4. 6 N H_2SO_4 : Dilute 18 ml of concentrated sulfuric acid to 108 ml.
5. Reagent C : Mix 1 volume of 6 N H_2SO_4 with 2 volumes of distilled water and 1 volume of 2. 5 % ammoniummolybdate, then add 1 volume of 10% ascorbic acid and mix well (prepare fresh each day).

Procedure:

Preparation of samples : Dilute urine with distilled water (if clear) or dilute HCl (if turbid). If the approximate range is unknown, run several dilutions.

Add 0. 5 ml of whole blood, plasma or serum to 2 ml of 10% TCA. Mix well, centrifuge and pipette off the filtrate. For routine analysis use 0. 5 ml of filtrate.

Pipette the phosphorus standards, diluted urine, ashed sample of biological material or TCA filtrate (blood plasma or serum) containing upto 8 μg of phosphorus into a 15 ml graduated centrifuge tube and adjust the volume to 4 ml with distilled water. The reagent blank consists of 4 ml distilled water.

Pipette 4 ml of reagent C into each tube, cap with parafilm, mix and place the rack with all tubes at 37 C for 1.5–2 hr. Remove, allow a few min to cool to room temperature and read absorbance in Beckman DU spectrophotometer at 820 nm against the blank.

Reference :

Anal. Chem. **28**, 1756 (1956).

6. IRON

a. Wong's method

Principle :

Iron is determined colorimetrically making use of the fact that ferric iron gives a blood red colour with potassium thiocyanate.

Reagents :

1. 30 % H_2SO_4

2. 7 % Potassium persulphate solution : 7 g potassium persulphate is dissolved in glass distilled water and the solution made upto 100 ml.

3. 40% Potassium thiocyanate solution : 40 g KCNS is dissolved in 90 ml glass distilled water, 4 ml acetone added and the volume made upto 100 ml.

4. Standard iron solution : 702.2 mg ferrous ammonium sulphate is dissolved in 100 ml glass distilled water and after addition of 5 ml of 1 : 1 HCl, the solution is made upto 1 l and mixed thoroughly (0. 1 mg Fe/ml). The standard solution is prepared fresh once in 6 months.

Working standard solution (10 μg Fe/ml) is prepared by diluting the above solution 10 fold.

Procedure :

Food iron :

To an aliquot (6.5 ml or less) of the mineral solution enough water is added (if necessary) to make upto a volume of 6.5 ml followed by 1.0 ml of 30% H_2SO_4, 1.0 ml potassium persulphate solution and 1.5 ml 40% KCNS solution. The red colour that develops is measured within 20 min at 540 nm.

Haemoglobin iron :

Two ml of conc. H_2SO_4 is taken in a 50 ml volumetric flask. Add exactly 0.5 ml of well mixed blood, mix and to this add 2 ml of potassium persulphate, agitate the flask, cool and dilute with about 25 ml distilled water. Then 2 ml of sodium tungstate is added and the volume made upto the mark. Filter using Whatman No. 42 filter paper. Transfer 15 ml of the filtrate to a fresh tube, add 1 ml of potassium persulphate and 4 ml of potassium thiocyanate. Mix and read the colour at 540 nm in a colorimeter. A standard (10-100 μg) is run similarly and a standard graph is prepared.

Calculation :

g Haemoglobin = 0.3 x mg Fe.

Note :

If use of reagent containing traces of iron cannot be avoided, it should be seen that the final soutions of standards and test contain identical quantities of these reagents. Potassium thiocyanate should be added just before taking the readings.

Reference :

J. Biol. Chem. **77**, 409 (1928).

. Bathophenanthroline method

Principle :

Iron in serum is present as Fe^{+++} bound to transferrin. In estimations of serum iron the iron is eleased by mild acid treatment, the proteins are precipitated and the released iron (Fe^{+++}) is reduced o Fe^{++} by reducing agents and then this Fe^{++} is reacted with bathophenanthroline to form a pink colour omplex, whose intensity can be measured at 540 nm.

Reagents :

1. Protein precipitant : Aqueous solution made to contain 100 g TCA, 30 ml of thioglycolic acid nd 2 ml HCl per l. This should be stored in a dark brown bottle and is stable for atleast 2 months.

2. Chromogen solution : Sodium acetate (2 M) containing 250 mg bathophenanthroline sulfonate er l.

3. Iron standard solution, Stock solution : 70.2 mg of ferrous ammonium sulphate, $FeSO_4 (NH_4)_2$ $O_4.6H_2O$, dissolved in water containing 0.2 ml of 2 N H_2SO_4 and made upto 1 l. The iron concentra- on of the solution is 1 mg per 100 ml.

Working standard : Forty ml of stock solution is diluted to 100 ml with glass distilled water. his working standard contains 400 μg/100 ml.

Procedure :

To 1 ml of plasma is added 1 ml of water and 2 ml of the protein precipitant solution. Mixed horoughly and allowed to stand for 5 min and centrifuged to get an optically clear supernatant solution 2000 rpm for 15 min). Two ml of the clear supernatant is transferred to a clean tube and 2 ml of the hromogen solution added. Mixed and allowed to stand for atleast 5 min. The OD of the colour eveloped is measured in a photoelectric colorimeter using 54 filter or in a spectrophotometer at 535 nm. A reagent blank is prepared by substituting water for serum. The OD of the blank should not exceed .015 against distilled water in a 1 cm pathway. A standard curve is constructed using 0.1, 0.5, 1.0, .5 and 2.0 ml of working standard and treating them similar to the test sample.

Calculation :

X-amount of iron is present in 0.5 ml plasma. This value when multiplied by 200 would give a lasma iron concentration in μg per 100 ml serum.

Note :

All glassware to be used should be cleaned thoroughly by soaking in HCl or H_2SO_4 - sodium ichromate solution for 6 hr and rinsing it free from acid in repeated changes of iron free water. All hrough the estimation use only glass distilled water. Haemolysed sample should not be used for lasma iron determination.

Reference :

Br. J. Haematol. **20**, 451 (1971).

c. Absorption using a whole body counter

Principle :

The isotope ^{59}Fe with a half life of about 45 days emits gamma radiation of 1. 1 and 1. 3 meV and β radiation of 0. 46 and 0. 27 meV and is very useful in the measurement of iorn absorption using a whole body counter. The technique of whole body counting allows the measurement of iron absorption by direct measurement of retained radioactivity.

The whole body counter :

It consists of 2 identical Nal (Tl) detectors mounted on a steel frame work, one located above and one below the body of the reclining subject. Lead shielding weighing 225 Kg is provided around each crystal which cuts off radiation from all sides excepting the subject's side.

A wooden bed rolls on rails so as to transport the body in between the two detectors during a 10 min measurement period through a distance of 280 cm.

The drive is accomplished by a synchronous motor operating through a gear system that transports in either direction.

Light supports are used so as to elevate the head above the bed by 3 cm, the legs by 6 cm, and the arm by 8 cm. These ensure that the upper and lower surfaces of the adult body are equidistant.

The pulses from the detector photomultipliers are amplified and fed to a single channel spectrometer and finally registered by a scaler.

Instrument settings :

H. V.	—	1020 V
Lower discriminator	—	1. 34
Upper discriminator	—	8. 50
Timer preset to	—	10 min
Scaler preset to	—	maximum counts

With these settings, the smallest ^{59}Fe activity that can be assayed in an iron absorption test is 0. 1 μCi with 10% coefficent of variation (sensitivity=20 counts/10^4 γ-rays emitted).

Procedure :

Background : The electronics is switched on and allowed to warm up for an hr and the above mentioned settings are obtained.

The subject is made to recline on the bed in a supine position and centered on the bed. The body supports are kept in position to raise the arms, head and legs. The scaler is reset.

Then the drive switch is operated for a forward drive and simultaneously the scaler is started. A 10 min count is accumulated to obtain the background. A scan is made with the drive switch reversed and a second reading of background obtained to calculate a mean subject background. A mean instrument background is obtained by scanning the bed with the body supports in their usual position but without a subject lying on the bed.

(i) Elemental iron absorption : In this case, the tracer (3μ Ci of ^{59}Fe) is administered with adequate amounts of carrier iron in the form of a suitable salt preparation on an empty stomach after an overnight fast.

(ii) Food iron absorption :

(A) By the spiking technique : In this case 3 μCi of ^{59}Fe is administered along with breakfast after an overnight fast, the tracer being swallowed midway through the meal or preferably premixed with the meal before cooking.

(B) By use of labelled foods : The activity is biosynthetically incorporated into the food which is mixed with unlabelled food and administered to the subject.

In each case, no food is allowed for 3 hr after administration of the isotope. The subjects are scanned on the whole body counter to obtain the initial dose administered. Standard 3 μCi of ^{59}Fe placed on a polythene bottle is centered on the bed and scanned on the counter.

Twelve days after administration of isotope (time required for the administered activity to appear in circulation) the subjects are scanned once again on the counter to obtain the amount of activity retained in the body. The 3 μCi of ^{59}Fe standard is scanned again to compute the physical decay of the isotope in the body. Instrument background is also measured.

Calculations

Let,

B_i = Instrument background on the day of measurement of subject background.
B_s = Subject background.
B_{ii} = Instrument background on the day of administration of isotope.
P_i = Subject counts immediately after dosing.
S_i = Standard counts on the day of administration of isotope.
B_f = Instrument background on the 12th day after administration of isotope.
P_f = Subject counts 12 days after dosing.
S_f = Standard counts 12 days after administration of isotope.

then,

$$\% \text{ iron absorption} = \frac{\left(P_s - \frac{B_s \times B_f}{B_i}\right) \times \left(\frac{S_i - B_{ii}}{S_f - B_f}\right)}{\left(P_i - \frac{B_s \times B_{ii}}{B_i}\right)} \times 100$$

Reference :

Br. J. Haematol. **22**, 281 (1972).

d. Total iron binding capacity

Principle :

Transferrin is saturated 100 % by adding iron from out side in Fe^{+++} form. After chelating the iron not bound to transferrin the transferrin iron is estimated as in the case of serum iron.

Transferrin normally is saturated to only 33% by iron. Determination of transferrin saturation provides a good index of iron nutritional status.

Reagents:

1. Ferric chloride solution containing 5 μ g iron/ml in 0.005 N HCl.
2. Reagents as for iron estimation by bathophenanthroline method.

Procedure :

To 1 ml of serum is added 2 ml of ferric chloride solution, shaken well and the mixture allowed to stand for 5 min. (This gives a total addition of 1000 μg iron to each 100 ml serum while unsaturated iron binding capacity of the serum ranges from 0 – 500 μg/100 ml in normal and pathological states). Two hundred mg magnesium carbonate is added and the tube agitated frequently and thoroughly for 45 min. The tube is centrifuged at 3000 rpm for 10 min and the iron content of the supernatant is determined using 1.5 ml aliquot.

Calculation :

X – μg amount iron is present in 0.25 ml of serum. This value when multiplied by 400 would give TIBC value in μ g/100 ml of serum.

Unsaturated iron binding capacity (UIBC) = TIBC – Serum iron (in μg/100 ml serum).

Reference :

Advances in Clinical Chemistry. Vol. I, Acad. Press, p. 17 (1958).

7. MAGNESIUM

Principle :

Magnesium is converted to magnesium pyrophosphate, which is estimated gravimetrically.

Reagents :

1. 10% Ammonium phosphate solution.
2. 10% Sodium citrate solution.
3. 0.1% Methyl red indicator.
4. 1 : 4 and 1 : 10, ammonia : water solution.

Procedure :

To the calcium free filtrate (obtained from the filtrate after precipitation of calcium as oxalate) is added 30 ml of conc. HNO_3 and the solution evaporated completely on a boiling water bath. Five ml of conc. HCl and 100 ml of water are then added and the solution stirred well with a glass rod. It is followed by the addition of 10 ml of 10% ammonium phosphate solution and 5 ml of 10% sodium citrate solution and the mixture stirred. After adding 2 or 3 drops of methyl red indicator the solution is neutralised with the addition of 1 : 4 dilute ammonia. Strong ammonia (25 ml) is then added, stirred vigorously and the mixture left to stand overnight, filtered through Whatman No. 40 or 44 filter paper and washed free from chlorides using 1 : 10 dilute ammonia (tested with HNO_3+silver nitrate solution). The funnel with the precipitate on the filter paper, is dried in an oven. The filter paper is then transferred to a weighed crucible (the crucible is previously heated, cooled and weighed) and ashed slowly over a burner. It is then kept in a muffle furnace at 900°C for 2 hr. The crucible and the contents are cooled in a desiccator and weighed to get magnesium as its pyrophosphate.

Calculation :

$$\text{mg of magnesium/100 g of sample} = \text{weight of ash} \times \frac{48.64}{222.6} \times \frac{100}{\text{ash solution taken for estimation}} \times \frac{100}{\text{Wt of sample}} \times 1000.$$

8. SODIUM AND POTASSIUM IN BLOOD

Principle :

The sample in solution is introduced in the form of a fine continuous spray into a nonluminous gas flame. The emitted light, characteristic for the ion being analysed, is isolated and focussed on a photoelectric cell and the current intensity is measured on a suitable meter.

Reagents :

1. Isotonic glucose solution : 5.9 g glucose in 100 ml water.

2. Standard sodium solution : Prepare a stock standard solution containing 100 meq of sodium/l by dissolving 5.85 g of dried NaCl in glass distilled water and diluting it to 1 l. Working standards are prepared by taking 10 - 16 ml of the stock solution and making up the volume to 1 l. These standards represent 100 160 meq of Na per l at 1 : 100 dilution. A further 1 : 5 dilution is employed for all the working standard solutions so that the dilution factor for Na is 500.

3. Standard potassium solution : Prepare a stock standard solution containing 100 meq of K per l by dissolving 0.746 g of dried KCl in glass distilled water and diluting it to 1 l. Working standards are prepared by taking 15 - 35 ml of stock standard and diluting it to 1 l. These standards represent 3 - 7 meq K, at 1 in 20 dilution.

Procedure :

Plasma : One ml plasma (or serum) is made to 20 ml and aliquots taken for K determination. Dilution factor for this is 20. One ml of this diluted plasma is further diluted to 25 ml and used for Na estimation (dilution factor 500).

Erythrocyte : About 5 ml of blood is centrifuged in a clinical centrifuge in cold. The plasma is separated and the cells are washed with 4 - 5 ml isotonic glucose solution and again centrifuged in cold. This is repeated 2-3 times. The glucose solution is siphoned out as much as possible till a column of packed cells remains. One ml of the packed cells is made upto 50 ml with water and used for the estimation of erythrocyte Na. One ml aliquot of the above is further diluted to 4 ml and used for cell K. The same standard solution as above are used.

Calculations :

The sample Na or K is read by interpolation on the standard curves. For plasma (or serum) Na and K, the direct reading from the standard graph can be taken as the dilution factors in the sample and standards are the same.

For erythrocyte Na, 1/10th value is taken and for erythrocyte K, 10 times the value is taken. The results are expressed as meq of Na or K/ml.

Reference :

Hawk's Physiological Chemistry, Oser and Summerson (Eds.) 14th Edn. p. 1140 (1965).

9. CHLORIDES

Principle :

The chloride is quantitatively precipitated as silver chloride on adding silver nitrate. The amount

of silver nitrate used is quantitated titrimetrically against potassium thiocyanate.

Reagents :

1. 0.1 N NaOH
2. N/50 Silver nitrate
3. Ferric indicator : 20 g of ferric ammonium sulphate dissolved in 50 ml of water to which one or two drops of nitric acid is added.
4. N/100 Potassium thiocyanate

Procedure:

About 2-5 g of the sample is weighed into a boiling tube and 60 ml of 0.1 N NaOH is added, kept in a hot water bath and digested. The digestion is allowed to be completed and the mixture is made upto 100 ml. A 30 ml aliquot of the mixture is taken in a boiling tube and 5 ml of N/50 silver nitrate and 5 ml of HNO_3 are added and again digested on the water bath. When the volume of the digest is about 5 - 10 ml, 5 ml of HNO_3 is added continuing the digestion in the boiling water bath for another 30 min. Nitric acid fumes are allowed to escape. The tube is then cooled and 40 ml of distilled water, and 20 drops of (1.5 ml approximately) ferric indicator are added and the solution kept overnight in the cold. The next day, the contents are titrated against N/100 potassium thiocyanate. One reagent blank is run similarly along with the samples.

Calculation :

One ml of 0.01 N potassium thiocyanate = 0.335 mg of Cl.

$$\text{mg Cl/100g of sample} = \text{blank reading} - \text{sample reading} \times \frac{0.335}{0.01} \times \text{N of potassium thiocyanate} \times \frac{100}{30} \times \frac{100}{Wt}$$

References :

1. J. Biol. Chem. **58**, 523 (1923).
2. J. Biol. Chem. **79**, 221 (1928).

10. SULPHUR

Principle :

Sulphur is precipitated with barium chloride and is estimated gravimetrically.

Reagents :

1. 0.1% Methyl orange
2. 40% NaOH
3. 5% Barium chloride

Procedure :

To 2 - 3 g of sample weighed into a micro-Kjeldahl flask is added 5 ml of conc. HNO_3 and the flask is covered with glass stopper. The flask is then kept in a boiling water bath for 10 hr and then on a sand bath and digested on a low flame till a liquid digest results (additional nitric acid is added

if necessary). To this solution, 2 ml of perchloric acid is added and the temperature is raised. Addition of nitric acid and perchloric acid are repeated till the digest turns into a clear solution. Digestion is continued for a further 10–16 hr. By this time all the nitric acid would have escaped and the remaining clear solution can be transferred into a 500 ml beaker by repeated washings with distilled water to make approximately 200 ml. One or two drops of methyl orange indicator are added and the solution neutralised with the addition of 40% NaOH. The solution is again acidified with HCl and boiled for 5 min. Ten ml of 5% barium chloride is added and the solution boiled for 5 min. It is kept overnight and then filtered through Whatman No. 40 or 44 filter paper and washed with distilled water till free from barium chloride. The precipitate along with the filter paper is kept in an oven for drying. The filter is then taken in a weighed crucible and ignited, taking care to avoid spurting and finally ashed at 600°C for 3 – 4 hr and weighed.

Calculation :

$$\text{g of sulphur/100 of sample} = \text{Wt of the ash} \times 0.1374 \times \frac{100}{\text{Wt of sample}}.$$

Reference :

Biochem. J. **33**, 1313 (1939).

11. FLUORINE

Principle :

Determination of micro quantities of fluorine is based on the stoichiometry of the reaction between hydrofluosilicic acid and thorium nitrate under specific experimental conditions. The total fluorine in the known amount of the material is converted to the inorganic form by ashing, it is then distilled with H_2SO_4 and silicon carbide as hydrofluosilicic acid and finally titrated with standard thorium nitrate using alizarin red as an indicator.

Reagents :

1. Stock standard, 1 mg fluorine/ml : 2. 210 g of sodium fluoride is dissolved in 1 l glass distilled water. Working standard (10 μg/ml) : One ml of the stock is diluted to 100 ml in a standard volumetirc flask.
2. 0. 2 mM Thorium nitrate : 300 mg of thorium nitrate is dissolved in 100 ml of glass distilled water. The exact molarity of the solution is determined by estimating thorium as thorium dioxide in this solution. The volume of the stock solution is properly adjusted so as to get 0.2 mM thorium nitrate solution.
3. Monochloroacetic acid – NaOH buffer (pH 3.0) : 0. 02 M solution of monochloroacetic acid is prepared in water and adjusted to pH 3.0 with NaOH.
4. 50 % Sulphuric acid (V/V) : The acid should be boiled to make it free from sulphur dioxide.
5. Glass beads and silicon carbide : These should be made fluorine free by boiling with 30 % H_2SO_4 for a sufficient length of time.
6. Ashing agent : One g of calcium oxide, 1 g of magnesium oxide and 2 g of magnesium acetate are dissolved in water and made upto 100 ml.
7. Indicator : Sodium alizarin sulfonate 0.05 % in water.

Procedure :

Ashing : A suitable amount of sample (fluorine content approximately 15-30 μg) is weighed in a platinum crucible and macerated with 15 ml of ashing agent. The mass is dried over a water bath and then in an oven at 100°C. The dried material is ignited gently over bunsen burner and finally ashed in a furnace at 500°C.

While estimating fluorine in water sample, it is necessary initially to concentrate it to small volume.

Distillation of fluorine as hydrofluosilicic acid : The ashed material is transferred quantitatively with 40 ml of 50% H_2SO_4 to a distillation flask of 125 ml capacity, containing two glass beads and 0.5 g of silicon carbide. The flask is fitted with a thermometer and a thistle funnel, their tips reaching within half an inch of the bottom of the flask. The distillation flask is attached to a water condenser in a vertical position and the lower tip of the condenser is immersed in a few ml of water in the receiver. The flask is then heated with a burner over a wire gauze and the distillation is carried out at 114°C. The temperature is maintained by properly adjusting the flame. The volume of liquid in the distillation flask is maintained constant by adjusting the flow of water from the thistle funnel. The distillation is continued exactly for 60 min at desired temperature and the volume of the distillate is noted.

Titration of hydrofluosilicic acid with thorium nitrate : Ten ml aliquots of the distillate are pipetted out in two volumetric flasks. The pH of one of them is determined (usually less than 3.0) and adjusted exactly to 3.1 by the addition of 0.02 M NaOH. The volume of NaOH needed for this pH adjustment is noted and equivalent quantity of the alkali is added to the other flask, to which is further added 2.0 ml of monochloroacetic acid buffer. The solution is then titrated against 0.2 mM thorium nitrate solution till the appearance of a pink colour, using alizarin red as the indicator.

Calculation :

A standard curve is prepared following the above procedure with standard NaF solution ranging from 10 – 50 μg. A blank is run with glass distilled water under identical conditions with each set of experiment. The titre value for the sample after subtracting the blank value, is read on the standard curve and the amount of fluorine in the sample is calculated.

Note :

1. Analysis is done in duplicate using 100 ml water for each estimation and for each mineral.
2. Water samples are best collected in clean, polythene reagent bottles with tight fitting polythene covers and screw cap tops.
3. A few crystals of thymol (about 10 mg) are added to each sample of water to prevent any fungal growth.

Reference :

J. Ind. Inst. Science **34**, 135 (1952).

12. IODINE

Principle :

Quantitative determination of micro amounts of iodine is based on catalytic reduction of ceric (Ce^{++++}) ion to cerous (Ce^{+++}) ion by iodine.

Reagents:

1. Sodium carbonate–potassium chloride solution : 212 g of anhydrous Na_2CO_3 and 20 g of $KClO_3$ are dissolved in water and the volume made up to 1 l.
2. 0.02N Arsenious acid reagent : 0.986 g AsO_3 is dissolved in 10 ml of 0.5N NaOH in a beaker by heating. This is added to 850 ml of water and to this 20 ml HCl and 20.6 ml H_2SO_4 are added. The volume is then made upto 1 l.
3. 0.03N Ceric ammonium sulphate : 48.6 ml conc. H_2SO_4 is added to about 600 ml of water and then 20 g of ceric ammonium sulphate is dissolved in this solution. The total volume is then made upto 1 l with water.
4. Standard KI solution : A stock solution containing 4 μg of iodine per ml is prepared in water and the working standards are prepared as follows : 0,1.0, 2.0, 3.0and 4.0 ml aliquots of the stock solution are taken and the volume is made upto 100 ml with water. These solutions thus contain 0, 0.04, 0.08, 0.12 and 0.16 μg iodine per ml.

Procedure:

Digestion : A suitable amount of sample (usually containing 0.04–0.08 μg of iodine) is taken in a pyrex test tube (15 x 125 mm). Na_2CO_3-$KClO_3$ reagent 0.5 ml is added and the contents are dried at 110C°. Complete drying takes about 2 hr.

Incineration : The above test tubes are then transferred to thermostatically controlled furnace and incineration is carried out at 610°C for 30 min.

Extraction : The ashed material is extracted with 10 ml of arsenious acid solution at room temperature. It is allowed to leach for 15 min mixed and centrifuged for 20 min at 2000 rpm.

Colorimetry : Five ml supernatant is pipetted out in a test tube immersed in a water bath maintaind at 37°C. After the tube has reached the temperature, 1.0 ml of ceric ammonium sulphate is added and exactly after 20 min the reduction of ceric to cerous is read in a photoelectric colorimeter at 420 nm. Standard solution of KI containing 0.0 – 0.16 μg of iodine per ml is run simultaneously.

Calculation:

A straight line response is obtained by plotting concentration of iodine in μg against readings on Klett – Summerson photoelectric colorimeter on a semilogarithmic scale (ie. Klett readings on logarithmic scale and concentration on cm scale). Using this standard graph the values for any unknown sample is read.

Note:

For each set of samples, a fresh standard curve should be obtained, since a day to day variation has been observed in the readings for standard.

References:

Clinical Chemistry, Principles and Techniques, R. J. Henry (Ed.), Harper and Row Publishers, New York, p. 921 (1964).

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VIII. HORMONES

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1. PROTEIN HORMONES

a. Insulin – RIA

Iodination of insulin :

Reagents :

1. 0.075 M Barbital buffer, pH 8.6 : Dissolve 15.464 g sodium barbitone in about 800 ml water. Add 30 ml of 0.3 M HCl, mix and adjust pH to 8.6. Make up the volume to 1 l with water. Add 100 mg merthiolate as preservative.

2. Buffer A – 0.3 M Phosphate buffer, pH 7.5 : Mix together 16.4 ml of 0.3 M Na_2HPO_4 (4.26 g in 100 ml) and 3.6 ml 0.3 M NaH_2PO_4. 2 H_2O (4.68 g in 100 ml).

3. Buffer B – 0.03 M Phosphate buffer, pH 7.5 : Take 5 ml buffer A and dilute to 50 ml with water.

4. Insulin solution : Weigh exactly 50 μg pure crystalline pork insulin and dissolve in 100 μ l of buffer B just before use.

5. Chloramine T : Dissolve 12.5 mg chloramine T in 5 ml of buffer B just before use.

6. Sodium metabisulphite : Dissolve 25 mg sodium metabisulphite in 5 ml of buffer B just before use.

7. Albumin solution A : Dissolve 125 mg bovine serum albumin (BSA fraction V) in 5 ml buffer B.

8. Albumin solution B : Dissolve 2 g BSA in 100 ml barbital buffer.

9. Radioactive iodine : Carrier free ^{125}I as sodium iodide is purchased from Radiochemical Centre, Amersham, U. K. The specific activity is about 10 mCi/0.1ml.

10. BSA - borate buffer : Dissolve 8.25 g of boric acid and 2.7 g of NaOH in about 900 ml water. Adjust pH to 8.0 with 12 N HCl and make up the volume to 1 l with water. Add 100 mg merthiolate as preservative. Dissolve 5.0 g of BSA in 100 ml of the buffer.

Preparation of sephadex column : Add 1 g of sephadex G – 25 to about 50 ml of 0.075 M barbital buffer, mix well and let stand for 30 min. Take a 1 x 20 cm glass column and put some glass wool at the bottom. Pour the sephadex suspension to a height of 10 cm. Equilibrate the column with albumin by passing 20 ml of albumin solution B through it. The column should be used soon after preparation. A fresh column should be prepared each time.

Protocol for iodination :

Take 20 μ l of radioactive iodine (2 mCi) in a clean test tube (3 x 0.5cm) and count its radioactivity in a suitable gamma counter.

Add the following reagents in sequence given below : Buffer A – 20 μ l, insulin solution 10 μ l and chloramine T solution 5 μ l. Mix on a vortex for exactly 30 sec and then add sodium metabisulphite 5 μ l, mix for 5 sec and add 50 μ l of albumin solution A.

Transfer the reaction mixture on to the sephadex G – 25 column by means of a capillary pipette. Add another 50 μ l of albumin solution A to the reaction vial, mix and transfer on to the column. Count the residual radioactivity in the empty reaction vial along with the pipette. Elute the column with barbital buffer and collect 2 ml fractions at the rate of 1 ml/1.5 min. Collect 20 such fractions and count the radioactivity. A typical elution gives two peaks, a protein peak and a salt peak. The protein peak appears at 4 – 6 ml and the salt (iodine) peak at 9–12 ml.

Purification of ^{125}I insulin :

Take the eluate containing the protein fraction with the maximum radioactivity (usually the 6th ml fraction) for further purification. During iodination some of the insulin molecules may get damaged and are broken down into smaller fragments. They can be separated from the intact iodinated insulin molecules by making use of their property of binding to plasma proteins.

Prepare a suspension of sephadex G – 75 as described for sephadex G – 25. Pack a glass column (1 x 20 cm) to a height of 15 cm. Take 0.1 ml of the eluate chosen for purification and add 0.1 ml of fresh normal human plasma. Transfer the mixture to the sephadex column. The elution of the column, collection of eluate and counting is the same as described earlier.

Two peaks appear in the eluate. The first peak representing damaged insulin appears at 6 – 7 ml and the second one representing intact insulin at 11 – 13 ml. Take the eluate from the second peak which gives the maximum radioactivity and dilute with borate buffer, pH 8.0 containing 5% BSA (see assay procedure), so that 0.1 ml contains about 50 – 100 pg insulin and atleast 10,000 cpm.

Preparation of insulin antiserum :

Dissolve 2 μg pork insulin in 0.5 ml of 0.03 N HCl and add 2.0 ml water. Take this in a 10 ml syringe. Take 2.5 ml Freund's adjuvant in another syringe. Join the nozzles of both syringes with a short teflon tube. Prepare an emulsion by repeatedly pushing the contents of one syringe into the other. The emulsion is ready when a drop put in water does not spread.

Take young adult guinea pigs (300 – 500 g) and inject 1 ml of the emulsion (contains about 10 units insulin) subcutaneously into each animal, either on the abdomen or on the inner side of the thigh. Give weekly injections for 4 weeks. Bleed the animals 10 days after the last injection by cardiac puncture. Separate the serum and dilute 100 times with 5% BSA in borate buffer. Dispense aliquots in separate vials and lyophilise. Store in the cold.

Preparation of rabbit anti-guinea pig globulin serum (precipitating serum) :

Preparation of gammaglobulins : Prepare a saturated solution of ammonium sulphate 5.35 M(70%) at 0°C or 5.75 M (76%) at room temperature. Add 5 ml of this to 10 ml guinea pig serum. Centrifuge and dissolve the precipitated proteins in about 5 ml water. Add 2.5 ml of the saturated ammonium sulphate solution. Centrifuge and throw off the supernatant. Repeat this 4-5 times. The globulin concentrate thus obtained should contain 95% protein when estimated by the biuret reaction (protein should not be estimated by the micro – Kjeldahl procedure since $(NH_4)_2SO_4$ is present in the precipitate). The precipitate may be dissolved in 5 ml water and dialysed at room temperature for 3–4 hr to remove $(NH_4)_2SO_4$ and then lyophilised.

On the day of the injection, prepare an emulsion of the gammaglobulin in Freund's complete adjuvant so that 1 ml of the final emulsion will contain 1 mg gammaglobulin.

Take 4 or 6 healthy adult rabbits and inject 1 ml of the emulsion subcutaneously into each rabbit. Repeat the injection weekly for 4 weeks. Ten days after the last injection, bleed the animals. Separate and store frozen.

Procedure :

Standard insulin solution, first stock solution (200 μg/ml or 5 units/ml) : Dissolve 2 mg pork insulin in 1 ml of 0.03 N HCl and make up the volume to 10 ml with glass distilled water. Dispense 0.1 ml aliquots into separate vials and store frozen at – 20°C. Further dilute 0.1 ml of the first stock solution to 10 ml with assay buffer (2 μg/ml or 50 m units/ml).

Working standard (16 ng/ml or 400 μ units/ml) : Dilute 0.2 ml of the diluted standard (2 μg/ml) to 25 ml with assay buffer and make various dilutions as follows from this solution :

Standard No	Insulin	Assay buffer (ml)	Final insulin concentration	
			ng/ml	μ units/ml
1.	1.0 ml working standard	9.0	1.6	40.0
2.	3.0 ml of No. 1	3.0	0.8	20.0
3.	3.0 ml of No. 1	5.0	0.6	15.0

Standard No	Insulin	Assay buffer (ml)	Final insulin concentration	
			ng/ml	μ units/ml
4.	3.0 ml of No. 2	3.0	0.4	10.0
5.	2.0 ml of No. 3	2.0	0.3	7.5
6.	0.3 ml of No. 1	2.7	0.16	4.0
7.	0.3 ml of No. 2	2.7	0.08	2.0
8.	0.5 ml of No. 4	4.5	0.04	1.0
9.	2.0 ml of No. 8	2.0	0.02	0.5

Guinea pig anti-insulin serum : The dilution of the antibody used is usually around 1 in 20,000 (optimum dilution to get about 40% binding with antigen has to be worked out for each batch).

Rabbit anti-guinea pig globulin serum (precipitating serum) : Optimum dilution to get maximum precipitation has to be worked out for each batch but usually 1 in 2 dilution is used.

Normal guinea pig serum : Bleed a normal guinea pig by cardiac puncture and separate the serum. Store the serum in small aliquots at – 20°C. A fresh vial is taken every time and diluted 300 times with assay buffer.

Assay :

Pork insulin – ^{125}I, 50-100 pg	— 0.1 ml
Standard pork insulin or serum after appropriate dilution	— 1.0 ml
Anti-insulin serum (1 : 20,000)	— 0.1 ml
Mix, incubate for 24 hr at 4°C and add	
Rabbit anti-guinea pig globulin serum (1 in 4 dilution)	— 0.1 ml
Normal guinea pig serum (1 : 300)	— 0.1 ml

Incubate for further 24 hr at 4°C. Centrifuge the tubes at 3000 rpm for 30 min at 4°C. Decant the supernatant from each tube carefully without disturbing the precipitate. Invert the tubes on a filter paper for some time and wipe off the sides. Count the precipitate (bound ^{125}I insulin) in a well type scintillation counter.

Calculation :

Calculate the Ci/Co x 100 (Ci – counts in precipitate with added standard insulin; Co-counts in precipitate without standard insulin). Plot the Ci/Co values against concentration of insulin.

Note :

The dilution of precipitating serum should be such that the precipitate should be easily visible with the naked eye and should not get disturbed while the supernatant is being decanted.

Serum or plasma insulin levels are estimated by replacing the standard insulin by serum or plasma in the incubation tube.

References :

1. Biochem. J. **89**, 114 (1963).
2. Diabetes, **14**, 771 (1965).

b. Growth hormone–RIA

Iodination of human growth hormone :

The reagents and procedure for iodination of human growth hormone (HGH) are similar to those described for iodination of insulin except that pure HGH is substituted for insulin. For separation of the iodinated HGH, sephadex G-50 is used. The protein peak appears in the 3rd or 4th ml of eluate and iodide peak at 9-10 ml.

Purification of ^{125}I HGH:

The procedure of purification is the same as described for insulin except that the separation of the broken ^{125}I HGH from the intact hormone is achieved on sephadex G–100 (1 x 50 cm). The peak of the broken hormone appears at 14th ml and that of intact ^{125}I HGH at 28 – 30 ml.

Procedure :

All dilutions of standard human growth hormone, ^{125}I HGH, guinea pig anti-growth hormone serum, normal guinea pig serum and precipitating serum are made with borate buffer containing 0.1% BSA and 0.01% merthiolate.

Standard HGH solution, Stock solution (5 μg/ml) : Dissolve 0.1 mg HGH in minimum amount of 1 N NaOH and make up the volume to 20 ml with buffer.

Working standard (40 ng/ml) : Dilute 0.2 ml of the first stock solution to 25 ml with borate buffer or barbital buffer and make various dilutions as follows from this solution.

Standard No	HGH solution	Albumin-borate buffer (ml)	Final HGH concentration (ng/0.5 ml)
1.	1.0 ml working standard	4.0	4.0
2.	2.0 ml of No. 1	2.0	2.0
3.	2.0 ml of No. 2	2.0	1.0
4.	1.0 ml of No. 1	4.0	0.8
5.	2.0 ml of No. 4	2.0	0.4
6.	2.0 ml of No. 5	2.0	0.2
7.	2.0 ml of No. 6	2.0	0.1
8.	2.0 ml of No. 7	2.0	0.05

Guinea pig anti – HGH serum : The dilution of antibody used is usually around 1 in 150,000 (optimum dilution to get around 40% binding with antigen has to be worked out for each batch).

Rabbit anti-guinea pig globulin serum (precipitating serum) : This is prepared as described for insulin assay. Optimum dilution to get maximum precipitation has to be worked out for each batch but usually 1 : 2 dilution is used.

Normal guinea pig serum : 1 : 300 dilution

Assay :

Standard HGH or serum after appropriate dilution	—	0.5 ml
Anti – HGH serum (1 : 150,000)	—	0.1 ml

Mix, incubate at 37°C for4 – 6 hr and then add

^{125}I – HGH (6000 cpm/0.1 ml or 200 pg)	—	0.1 ml

Again mix and incubate at 37°C for 20 hr and add

Rabbit anti-guinea pig globulin serum (1 : 2)	—	0.1 ml
Normal guinea pig serum (1 : 300)	—	0.1 ml

Incubate for 20 hr at 4°C. Centrifuge the tubes at 3000 rpm for 30 min at 4°C. Decant the ernatant fluid from each tube carefully without disturbing the precipitate into another clean, rimmed : tube. Count the precipitate (bound ^{125}I–HGH) in a well type scintillation counter.

Calculation :

Calculate the Ci/Co x 100 (Ci – counts in precipitate with added standard HGH and Co–counts in cipitate without added standard HGH) and prepare a standard curve.

References :

1. Diabetologia. 3, 413 (1967).
2. J. Nucl. Biol. Med. 12, 137 (1968).
3. J. Nucl. Biol. Med. 13, 26 (1969).

Prolactin - RIA

Reagents :

1. Buffer A – 0.5 M Phosphate buffer, pH 7.6 : Mix 13.0 ml of 1.0 M NaH_2PO_4 H_2O (15.6 g in 0 ml water) and 87.0 ml of 1.0 M Na_2HPO_4 (71 g in 500 ml water) and dilute to 200 ml with water.
2. Buffer B – 0.05 M Phosphate buffer, pH 7.6 : Dilute buffer A 1 in 10.
3. Assay buffer : Dilute 20 ml buffer B to 100 ml with water. Add 0.9 g NaCl, 1 g BSA, 0.931 g TA and 100 mg sodium azide. Mix well till all are dissolved.
4. 0.05 M Carbonate-bicarbonate buffer, pH 9.5 : Mix 13 ml of 0.2 M Na_2CO_3 (2.12 g in 100 ml ater) and 37 ml of 0.2 M $NaHCO_3$ (1.68 g in 100 ml water). Dilute to 200 ml with water.
5. Human prolactin (obtained from National Institute of Health, USA): Weigh exactly 50 μg man prolactin and dissolve it in about 25 μl of the carbonate-bicarbonate buffer. Dilute to 50 μl with ffer B. This gives a concentration of 1 μg/μl.
6. Chloramine T : Dissolve 5 mg in 10 ml of buffer B.
7. Sodium metabisulphite : Dissolve 10 mg in 10 ml of buffer B.

Procedure :

Protocol for Iodination :

Human prolactin 5 μl, buffer A 25 μl, carrier free Na^{125}I 10 μl and chloramine T 10 μl are mixed n a vortex mixer for exactly 30 sec. Then add sodium metabisulphite 10 μl and normal human serum 00 μl.

Use a sephadex G - 50 column for separation of iodinated prolactin. Use buffer B for elution. The ormone peak appears at 7 ml and the salt peak at 21 ml.

Purification :

Same as described for HGH. Use buffer B for elution. The peak of the damaged hormone appears at 12 ml and that of the intact hormone at 22 ml.

All dilutions of standard prolactin, antiserum, precipitating serum and normal rabbit serum are made with the assay buffer.

Standard prolactin, stock (1 μg/0.1 ml) : Take 30 μl of prolactin solution, described earlier. Dilute with buffer A containing 1% BSA to 3 ml. Make further dilutions as follows, with assay buffer.

Stanadard No	Prolactin solution	Assay buffer (ml)	Final conc. (ng/0.1 ml)
1.	Stock 0.1 ml	1.9	50.0
2.	0.2 ml of No. 1	0.3	20.0
3.	0.2 ml of No. 1	0.8	10.0
4.	0.2 ml of No. 1	1.8	5.0
5.	0.3 ml of No. 3	0.7	3.0
6.	0.1 ml of No. 1	2.4	2.0
7.	0.2 ml of No. 5	0.2	1.5
8.	0.5 ml of No. 6	0.5	1.0
9.	0.3 ml of No. 8	0.3	0.5

Rabbit anti - human prolactin serum : Dilute to obtain about 40% binding with antigen.

Guinea pig anti-rabbit globulin serum (precipitating serum) : Refer insulin - RIA.

Assay :

Standard prolactin or serum after appropriate dilution	— 0.1 ml
Anti - prolactin serum	— 0.1 ml
Assay buffer	— 0.2 ml
Mix, incubate at 4°C for 3 days and then add,	
^{125} I - Prolactin (15, 000 cpm or 500 pg/0.1 ml)	— 0.1 ml
Mix and incubate for 2 days, add	
Guinea pig anti - rabbit globulin serum (1:2)	— 0.1 ml
Normal rabbit serum (1:100)	— 0.1 ml

Mix, incubate at 4°C for 20 – 24 hr and proceed as described for insulin assay.

Calculation :

Serum or plasma prolactin can be estimated by replacing the standard prolactin by serum or plasma in the incubation tube. Prolactin levels can be calculated from the standard curve.

References :

1. J. Clin. Endocrinol. Metab. **36**, 509 (1973).
2. Methods of Hormone Radioimmunoassay. Jaffe and Behrman (Eds.), Academic Press, 2nd Edn. (1979).

d. Somatomedin - bioassay

Principle :

The somatomedin activity of serum is measured by a bioassay, wherein the ability of cartilage to take up sulphate in the presence of serum is studied.

Reagents:

1. 0.9% NaCl solution
2. Eagle's medium : Dissolve 1 g of the lyophilised sample of the medium in 10 ml distilled water. Store in the cold.

Working solution(prepared on the day of the assay): To 1 ml of stock Eagle's medium, add 1 ml of an antibiotic solution containing 1750 IU of crystalline penicillin and 175 μg streptomycin. Add 8 ml distilled water.

3. Krebs - Eggleston's buffer, concentrated stock :

i. 2.72 g NaCl, 56.8 mg Na_2SO_4 and 160 mg KCl
ii. 63.3 mg $NaH_2PO_4.2H_2O$
iii. 80 mg $CaCl_2$
iv. 32 mg $MgCl_2$

Dissolve i - iv separately in 20 ml water and store.

v. Dissolve 4.4 g $NaHCO_3$ in 100 ml water. On the day of use, pass CO_2 through the solution till the pH reaches 7.4.

Working buffer : On the day of use, mix 1 ml each of solution i - v and add 15 ml distilled water.

4. 0.1 M Phosphate buffer, pH 6.5 : Mix 8 volumes of 1.56% $NaH_2PO_4.2H_2O$ and 5 volumes of 1.42% Na_2HPO_4. Check pH.
5. Enzyme solution : Dissolve 50 mg papain, 74.4 mg disodium EDTA and 35 mg cysteine hydrochloride monohydrate in 100 ml phosphate buffer.
6. Saturated solution of sodium sulphate.
7. $Na_2{}^{35}SO_4$ (carrier free, supplied by BARC) : Dilute with 0.9% NaCl to obtain 100 μCi/ml. On the day of use, dilute 1 ml of this with 4 ml of buffer to give 2 μCi/0 1 ml.
8. Bray's scintillator solution (Refer Appendix).

Procedure :

Fast male albino rats (21 - 27 days old) for 48 hr during which water may be allowed *ad libitum*. Kill and dissect out 5 long ribs from each side. Remove muscle and other tissues completely. Place each rib in a separate container with 0.9% NaCl. Keep in ice till the dissection is complete. Discard the first 6 mm of the cartilage from the vertebral end. Divide the remainder into 4 equal pieces (about 2 mm length).

Assay :

The assay should be carried out in 10 ml screwcapped test tubes. Each concentration of serum is set up in triplicate or quadriplicate.

Tube No	Eagle's medium (μl)	Reference serum (μl)	Test serum (μl)	Krebs-Eggleston's buffer (μl)
1.	200	30	—	370
2.	200	60	—	340
3.	200	—	30	370
4.	200	—	60	340
5.	200	90	—	310
6.	200	120	—	280
7.	200	—	90	310
8.	200	—	120	280

Add 0.1 ml $Na_2{}^{35}SO_4$ solution (2 μCi) to all the tubes. Incubate in a metabolic shaker at 37°C for 24 hr with continuous shaking at a frequency rate of 80 per min. Place the tubes in a boiling water bath for 8 min to arrest the reaction. Wash the cartilage pieces thoroughly under tap water. This is to remove any radioactivity present on the outside of the cartilage. Keep each piece in the saturated solution of sodium sulphate and allow to stay overnight. Wash the pieces thoroughly under tap water. Dry in a hot air oven at 100°C for 2 hr. Weigh the pieces on a torsion balance. Put each piece in 0.3 ml of enzyme solution and allow to stay overnight at 37°C. This helps in digesting the cartilage. Take 0.05 ml of the digest and add to 10 ml Bray's solution in a counting vial. Measure the radioactivity in a liquid scintillation counter.

Calculations :

The somatomedin activity of the test serum is expressed as potency ratio with respect to the reference serum which is arbitrarily given the value of 1.0. Dose response lines are constructed with log concentration of serum versus cpm per mg dry weight. The slope ' b ' is calculated for both the reference and the test serum from the dose response lines by the formula :

$$b = \frac{XY}{CSS_X}$$, where, X is log concentration of serum and Y is response as cpm/mg dry weight and CSS_X is corrected sum of square for X.

Slopes of the straight lines obtained with reference and test plasma are combined according to the equation given below to compute the pooled slope.

$$b_p = \frac{(XY)R + (XY)U}{(CSSx)R \times (CSSx)U}$$, where b_p = pooled slope and (XY)R and (XY)U refer to the corrected cross product for reference and unknown serum.

The potency of the unknown serum is calculated from the equation,

$$\text{Log potency} = X_U - X_R - \frac{Y_R - Y_U}{b_p}$$, where, X_U and X_R are mean log dose of unknown and reference serum and Y^U and Y^R are mean response of unknown and reference serum and b_p is the pooled slope.

Note :

Reference serum - Collect a large volume of blood from healthy donors. Serum from several donors may be pooled. Store frozen in 5 - 6 ml portions. This is done to avoid repeated thawing and freezing.

'est sera-Use serum or heparinised plasma. Store frozen till use. Can be stored upto 6 months. All glassware should be thoroughly cleaned and autoclaved at 15 lbs pressure for 15 min.

References:

1. Acta Endocrinologica, **57**, 557 (1968).
2. J. Clin. Endocrinol. Metab. **19**, 743 (1959).
3. J. Endocrinol. **54**, 365 (1972).
4. Biochem. J. **34**, 442 (1940).
5. J. Biol. Chem. **226**, 197 (1957).
6. Statistical Methods in Biological Assay, D. J. Finney (Ed.), (1971).

2. STEROID HORMONES

a. Cortisol

(i) Competitive protein binding assay

Principle:

The method is based on the competition between a steroid and a suitable tracer for the binding sites on a specific binding protein. The sample steroid displaces a portion of the tracer and the percentage of tracer bound to protein falls proportionally.

Reagents:

1. 1,2 - ^{3}H Corticosterone (Amersham, England): Dilute in ethanol to get a working solution of 10 μCi/ml.

2. Corticosteroid binding globulin (CBG): Collect venous blood from an adult male dog in heparinised tubes. Centrifuge immediately and separate the plasma. The plasma is stable for 6 months if stored at - 20°C.

3. Preparation of CBG - ^{3}H corticosterone mixture: Take 0.4 ml of ^{3}H corticosterone. Add about 90 ml distilled water and 2.5 ml of dog plasma. Make up the volume to 100 ml. The dilution of dog plasma to be used should be tested each time a fresh sample is obtained and a dilution which binds about 80% of the tracer steroid should be employed.

4. Preparation of florisil: Wash florisil 4 times by suspending it in water and allowing it to settle. Decant the water each time. The fine particles which do not settle down easily are thus discarded. Dry the florisil at 100°C for 1 hr.

5. Cortisol standard (Stock): Weigh 1 mg of the standard cortisol accurately. Dissolve in ethanol and make up the volume with the same solvent to 10 ml in a volumetric flask. This solution is stable for atleast 6 months when stored at 4°C. Dilute 1 ml of this stock solution to 100 ml with ethanol in a volumetric flask. Store at 4°C (1 μg/ml).

Working standard, 10 ng/ml: Dilute 0.1 ml of diluted stock (1 μg/ml) to 10 ml with ethanol in a volumetric flask. Store in the same manner as the stock solution.

6. Bray's scintillator solution: Refer Appendix.

Procedure:

Take 0.2 ml ethanol in centrifuge tubes. Add 5 and 10 μl test plasma to each of two tubes and

mix. The proteins of the plasma are precipitated by ethanol. Centrifuge for 10 min and transfer the supernatant into another tube. Wash the precipitate with another 0.2 ml of ethanol. Mix and centrifuge. Combine the two supernatants. Evaporate to dryness under nitrogen or in a 40 – 45°C water bath.

Take 0.05, 0.10, 0.15, 0.20 and 0.30 ml of the working standard and evaporate to dryness. Add 1 ml CBG – ^3H corticosterone solution to all the tubes. Mix thoroughly. Set up a blank with 1 ml CBG mixture alone. Incubate the tubes at 45°C for 5 min in a water bath. Immediately transfer the tubes to an ice bath and keep for 20 min. Add 80 mg florisil to all the tubes. Shake the tubes for exactly 30 sec on a vortex mixer in cold. Return the tubes to the ice bath for 20 min. Decant the supernatant into a clean tube. Florisil adsorbs the free corticosterone and the supernatant now contains only the bound corticosterone.

Take 10 ml of Bray's solution in a scintillation counting vial. Add 0.5 ml of the supernatant and count the radioactivity in a liquid scintillation counter for 5 min. Calculate the Ci/Co x 100 (Ci – counts in supernatant of standard or test sample and Co-counts in supernatant of blank). Prepare a standard curve by plotting the Ci/Co values against the concentration of cortisol. Calculate the cortisol value in the test sample with the help of the standard curve.

References :

1. J. Clin. Endocrinol. Metab. **27**, 973 (1967).
2. Principles of Competitive Protein Binding Assays, W. D. Odell and W. H. Daughaday (Eds.). J. B. Lippincott Co. Philadelphia and Toranto (1971).

(ii) Fluorimetric method

Principle :

The method depends on tne specific fluorescence of 11 - hydroxysteroids in concentrated sulphuric acid.

Reagents :

1. Methylene chloride : Add 1 l methylene chloride to 100 ml conc H_2SO_4 and let stand for several days with occasional shaking. Wash with 100 ml conc. H_2SO_4, two hundred ml distilled water, 100 ml NaOH and twice more with 200 ml distilled water. Dry over anhydrous Na_2SO_4 for 24 hr. Distil and collect the 39 - 40°C fraction and store in dark.

2. Conc. H_2SO_4 : This should be absolutely pure, or else the fluorescent reagent will become yellow and blank value will be very high. Store in cold.

3. Aldehyde - free ethanol : Add 10 g zinc dust and 10 g KOH to 1 l alcohol. Boil under reflux for 6 hr and distil. This should not discolour when added to H_2SO_4. Store in cold.

4. Fluorescent reagent : Take 17 volumes of aldehyde – free alcohol in a conical flask and keep in ice. Add 33 volumes of conc. H_2SO_4 slowly, drop by drop from a clean burette. Keep rotating the flask while adding H_2SO_4. The reagent should be prepared just before use and should be kept in ice.

5. Standard cortisol (hydrocortisone), Stock solution (1 mg/ml) : Dissolve 50 mg cortisol in 50 ml purified ethanol. Dilute 1 ml of stock to 100 ml with distilled water (10 μg/ml).

Working standard (1 μg/ml) : Dilute 1 ml diluted stock (10 μg/ml) to 10 ml with distilled water. All the solutions are stable at 4°C

Procedure :

Take 2 ml plasma in a 250 ml conical flask (U). Take 2 ml water for blank (B). Take 0.5, 1 and 2 ml of working standard, corresponding to 0.5, 1 and 2 μg cortisol (S_1, S_2, S_3). Add 15 ml methylene chloride to all the flasks. Shake gently in a mechanical shaker for 20 min. Cover the mouths of the flask with aluminium foil to minimise evaporation. Decant the extract into a test tube. Discard the top aqueous phase. Cover the test tube either with a stopper or aluminium foil. Take 5 ml fluorescent reagent in a glass stoppered test tube. Add 10 ml of the methylene chloride extract. Shake vigorously for 20 sec. Add extracts at 1 – 2 min intervals and time exactly. Wait till the two layers separate and discard the top methylene chloride layer. Transfer the acid extract into the flourimeter tube. Take readings on a spectro-fluorimeter exactly after 20 min.

Measure fluorescence with excitation at 470 nm and emission at 530 nm. Before taking the reading take care to wipe the exterior of the fluorimeter tube thoroughly. Acid will corrode the cell compartment of the instrument.

Calculation :

$$\mu g \text{ Cortisol/100 ml plasma} = \frac{\text{Reading U} - \text{B}}{\text{Reading } S_2 - \text{B}} \times \frac{100}{2}$$

Note :

Collect blood in heparinised containers and keep in ice. If rubber caps are used for covering the containers, they should be completely wrapped with aluminium foil. Contamination of blood with rubber, gives a very high fluorescence. Separate plasma as soon as possible. Plasma can be stored at 4°C for 48 hr and for several weeks at -20°C. While separating plasma, take care to avoid contamination with rubber.

Reference :

J. Clin. Path. **15**, 374 (1962).

b. Progesterone

(i) RIA

Reagents :

1. Phosphate buffered saline, pH 7.0 : Dissolve 3.042 g NaH_2PO_4. $2H_2O$, 4.331 g Na_2HPO_4 and 9.0 g NaCl in 1 l water. Add 100 mg merthiolate as preservative.

2. Assay buffer : Dissolve 10 mg BSA in 100 ml of above buffer.

3. 1, 2, 6, 7, ^{3}H-Progesterone (sp. act. 80-100 Ci/m mole) : Purchased from Radiochemical Centre, Amersham, U. K. Dilute with benzne and store. Take appropriate volume containing approximately 1 μCi and evaporate the solvent by passing nitrogen. Dissolve the residue in 10 ml assay buffer. Incubate at 37°C for 1 hr and store in cold.

4. Progesterone antiserum : This is produced in rabbits against 11-hydroxy progesterone coupled to BSA. (The antiserum obtained as a gift from Population council, USA).

5. Dextran-charcoal suspension : Add 250 mg charcoal (Norit A) and 25 mg dextran T-70 (Pharmacia) to 100 ml phosphate buffered saline. Mix thoroughly to form a fine suspension. Store at 4°C. Shake thoroughly before use.

6. Toluene scintillator : Refer Appendix.

7. Progesterone standards, Stock solution (1 mg/ml) : Dissolve exactly 10 mg progesterone in ethanol and make up the volume to 10 ml with ethanol. Dilute 1 ml of stock to 10 ml with a 1:1 mixture of ethanol and assay buffer (100 μg/ml). Dilute 1 ml of 100 μg/ml to 10 ml with assay buffer (10 μg/ml). Make further dilutions as follows:

Standard (ml)	Assay buffer (ml)	Final conc. (ng/0.1 ml)
1. 1.0 working standard	9.0	1.0
2. 2.0 of No. 1	2.0	0.5
3. 0.3 of No. 1	0.7	0.3
4. 0.4 of No. 1	1.6	0.2
5. 1.0 of No. 4	1.0	0.1
6. 1.0 of No. 5	1.0	0.05
7. 1.0 of No. 6	1.0	0.025
8. 0.2 of No. 5	0.8	0.01

Procedure :

Sample processing : Take varying volumes of plasma or serum, ranging from 0.2 – 1.0 ml. Add 0.1 ml (about 1000 cpm) of ^{3}H progesterone. Mix and let stand at room temperature for 1 hr. Add 5 ml petroleum ether and shake thoroughly. Pipette out the solvent phase. Repeat extraction with another 5ml of petroleum ether. Pool the solvent extracts and evaporate the solvent by passing nitrogen. Dissolve the residue in 0.6-1.0 ml assay buffer. Incubate at 37°C for 30 min.

Assay :

Standard progesterone or serum extract — 0.1 ml
Assay buffer — 0.1 or 0.2ml (to make volume to 0.4ml)
Antiserum (1 : 20, 000) — 0.1 ml
Mix and incubate at room temperature for 1 hr and add
^{3}H Progesterone (8,000-10,000 cpm/0.1 ml) — 0.1 ml
Mix and incubate at 4°C for 15-20 hr. Then add
Dextran charcoal suspension — 0.1 ml
Mix, each tube for 30 sec and let stand for 5 min. Centrifuge at 3000 rpm at 4°C for 20 min.

Decant the supernatant carefully, not disturbing the charcoal, into vials containing 10 ml of toluene scintillator. Incubate at 40°C for 1 hr or leave overnight at room temperature. Count the radioactivity.

Calculation :

Calculate Ci/Co x 100. Plot the values against concentration of progesterone. Read the values for unknown off the graph.

Reference :

Contraception, 5, 129 (1972).

(ii) Competitive protein binding assay

Reagents :

1. Silca gel G

2. Florisil
3. 1,2-^{3}H Corticosterone (Amersham, England). This is supplied in ethanol.
4. Bray's mixture (Refer Appendix).
5. Corticosteroid binding globulin (CBG) : Prepared as described for cortisol assay.
6. Preparation of CBG-^{3}H corticosterone mixture : This should be prepared atleast one day before the start of the assay. Take an appropriate volume of the tritiated corticosterone solution containing 25 ng of the steroid and evaporate it to dryness under nitrogen. Redissolve in 250 μl of ethanol. Add 1 ml of dog plasma and 39 ml of water. Thus the dog plasma is used in a dilution of 1 in 40. The dilution of the dog plasma to be used should be tested each time a fresh sample is obtained and a dilution which binds about 80% of the tracer steroid should be employed. This mixture is stable for atleast one week when stored at 4°C.

7. Progesterone standards, first stock solution (100 μg/ml) : Weigh 1 mg of the standard progesterone accurately. Dissolve in ethanol and make up the volume with the same solvent to 10 ml in a volumetric flask. This is stable for atleast 6 months when stored at 4°C.

Working stock solution (1 μg/ml) : Dilute 0.1 ml of the first stock to 10 ml with ethanol in a volumetric flask. Store in the same manner as the stock solution.

Procedure :

Preparation of TLC plates : Refer section A under Chromatography.

Keep the plates in a chamber containing methanol and wait till the solvent completely rises to the top. Remove, let the solvent evaporate and dry the plates in a dry air oven at 110°C for 20 min. This should be done on the day of use or just a day earlier.

Extraction and isolation : Take 0.2 ml of plasma in a glass stoppered tube and add 5 ml petroleum ether. Shake the tube vigorously for 1 min. Aspirate the petroleum ether layer either entirely or take an appropriate aliquot, and evaporate to dryness under nitrogen.

Dissolve the petroleum ether extracts in a minimal amount of chloroform and apply them on the TLC plates about 2 cm from the base. Apply 10 μg of the standard progesterone (corresponding to 0.1 ml of first stock standard) in one of the lanes. Carry out ascending chromatography in the solvent system, ether-benzene (2 : 1) for 40 min. Locate the standard spot under UV light (this will be seen as a fluorescent spot). Scrape corresponding areas for the unknown samples. Add 3 ml ether and 1 ml water and shake well. Take out the ether layer and evaporate to dryness.

Assay :

Take 0.2, 0.5, 1.0, 2, 3 and 4 ml of the working standard and evaporate to dryness under nitrogen. Add 1 ml of CBG – ^{3}H corticosterone to all the tubes. To the blank add 1 ml CBG mixture and no progesterone. Incubate the tubes at 45°C for 5 min in a water bath. Immediately transfer the tubes to an ice bath and keep for 60 min. Add 80 mg florisil to all the tubes and shake for exactly 30 sec on a vortex mixer. Return the tubes to the ice bath for 30 min. Decant the supernatant into a clean tube. (Florisil adsorbs the free corticosterone). Take 10 ml of Bray's mixture in a scintillation counting vial. Add 0.5 ml of the supernatant and count the radioactivity in a liquid scintillation counter for 5 min. Prepare a standard curve by plotting radioactivity against the concentration of progesterone. Calculate the progesterone value with the help of the standard curve.

References :

1. J. Clin. Endocrinol. Metab. **27**, 973 (1967).

2. Ibid. **27**, 1167 (1967).
3. Ibid. **28**, 1385 (1968).
4. Acta Endocrinol. **63**, 225 (1970).

c. 17β - Estradiol - RIA

Reagents :

1. 2, 4, 6, 7 (n) ^{3}H-Estradiol (sp. act. 100 Ci/mole) : Purchased from Radiochemical Centre, Amersham, U. K.
2. Estradiol antiserum : This is produced in rabbits against 6-keto-estradiol coupled to BSA.
3. All other reagents are same as for RIA of progesterone.

Procedure :

The assay procedure is exactly similar to that of progesterone RIA. However, 0.2 ml dextran coated charcoal is used.

Reference :

Mol. Asp. Med. **1**, 58 (1976).

d. Total estrogen in pregnancy urine

Principle :

Urine is acid hydrolysed to free the steroids from their conjugates and extracted with the appropriate solvent. Colour development is carried on directly without applying any purification procedure which is needed for nonpregnant urine samples.

Reagents :

1. 2.2 N HCl
2. 26.5 N H_2SO_4
3. 2% Hydroquinone : Dissolve 200 mg of hydroquinone in 10 ml of distilled methanol. To be prepared fresh every time.
4. 2% p - Nitrophenol : Dissolve 2 g of p-nitrophenol in 100 ml chloroform. To be kept in the dark and can be used for 4 days from the day of preparation if kept in the cold room.
5. Standard : Dissolve 5 mg of estriol in 50 ml of methanol. This can be used as stock solution (5 mg/50 ml). Stable at 4°C for 6 months.

Working standard : Five ml of stock solution is made upto 50 ml with distilled water (10 μg/ml).

Procedure :

One ml of urine is added to 10 ml of 2.2 N HCl in stoppered tubes. Hydrolysis is done for 60 min in a boiling water bath. A blank is kept along with the tubes by boiling 10 ml of 2.2 N HCl and 1 ml of distilled water. After the hydrolysis, the tubes are cooled. The hydrolysate is extracted twice with 10 ml of ether by shaking the tubes for 15 min each time. The ether extract is transferred to fresh stoppered tubes and evaporated to dryness at 40°C.

The standard solutions in the range of 5, 10, 15 and 20 μg is pipetted into stoppered test tubes and evaporated along with the hydrolysed extract in the water bath.

To the dried residue as well as to the evaporated standard solutions, 0.2 ml of 2% hydroquinone is added followed by 1 ml of 26.5 N H_2SO_4. The tubes are placed in the boiling water bath for 40 min shaking the tubes every 10 min. They are cooled in an ice bath for 15 min. Add 1.5 ml of cold distilled water to all the tubes. After 15 min 4 ml of 2% p - nitrophenol solution is added to each tube and the tubes are vigorously shaken. After the frothing subsides, the lower layer is removed and readings are taken in Beckman spectrophotometer at 500, 537 and 574 nm after adjusting the blank to zero at each wavelength.

Calculations :

Allen's correction is applied ;

$$\text{Corrected OD} = OD_{537} - \frac{(OD_{500} + OD_{574})}{2}$$

$$\mu\text{g Estriol/day} = \frac{\mu\text{g of estriol in unknown urine}}{\text{volume of unknown taken}} \times \text{total volume of urine.}$$

Since it has been established in our laboratory that there is a correlation between random and 24 hr excretion of estrol in urine when expressed in terms of creatinine excretion, the values are expressed as mg/g creatinine.

Urinary estrogen excretion is calculated as follows :

$$\text{Urinary estrogen mg/g creatinine} = \frac{\mu\text{g estriol in unknown}}{\text{volume of urine taken for analysis}} \times \frac{\text{total volume of urine}}{\text{creatinine g in total volume}} \times \frac{1}{1000}$$

Note :

As low as 2 mg estrogen/24 hr can be estimated by this method. Urine is collected in polythene bottles without any preservative and kept at - 20°C until analysed Analysis is completed within one week of collection.

Reference :

Br. J. Obstet. Gynecol. **71**, 92 (1964).

e. Neutral 17-ketosteroids

Principle :

The sulphate and glucuronide conjugates of neutral 17 – ketosteroids in urine are hydrolysed by boiling in strong acid. The free steroids are extracted into an organic solvent. Compounds with a-CH_2CO group react with m-nitrobenzene in alkali to give a red coloured compound. When the ketone group is at the 17 position, the compound has an absorption maximum at 520 nm. With steroids having a ketone group at other positions, the colour development is less intense and the absorption maximum is different.

Reagents:

1. Petroleum ether-benzene mixture : Mix equal volumes of petroleum ether and benzene.
2. 5% KOH

3. 1% m-Dinitrobenzene (m-DNB): Add to 10 g of m-DNB, a large volume of warm ethanol and a little 2 N NaOH. Cool and add 2-3 volumes of water. Collect the m-DNB on a Buchner funnel, wash 2–3 times with water and recrystallise twice from ethanol. This must crystallise in nearly colourless needles. Dissolve 1.0 g recrystallised m-DNB in 100 ml aldehyde-free ethanol.

4. 5N KOH: Weigh approximately 30 g KOH and dissolve in 450 - 500 ml water. Take a small volume, dilute 40 50 times with water and titrate accurately against 0.1 N HCl. Adjust volume of stock solution accordingly to obtain 5N KOH.

5. Stock standard of dehydroisoandrosterone (DHIA), 1 mg/ml: Dissolve 50 mg DHIA in 50 ml ethanol.

Working standard (100 μg/ml): Dilute 10 ml of stock to 100 ml with ethanol.

Procedure:

Take 10 ml of urine in a 40 ml glass stoppered test tube. Add 0.5 ml of concentrated HCl. Cover the top of each tube with a marble and keep in a boiling water bath for 20 min. Remove, cool and extract with 25 ml of petroleum ether-benzene mixture. Shake for 5 10 sec. Take the upper solvent layer into a 250 ml separating funnel. Wash with 1/15 volume of 5% KOH. Discard the lower KOH layer (it is important to remove the KOH completely). Wash the solvent twice with 1/10 volume of water. Discard the water layer.

Pipette out 20 ml of the extract into a 30 to 40 ml glass stoppered tube and dry in a water bath (40–45°C). Wash down the extract from the wall of the tube with ethanol. To the dried residue, add 0.4 ml of 1% m DNB to dissolve the residue.

Take 0.3 ml of the working standard solution in duplicate. Evaporate to dryness and add 0.4 ml 1% m-DNB. Shake well. Add 0.2 ml of 5 N KOH to all the tubes. Mix well and incubate in the dark at room temperature (close to 25°C) for 90 min. Add 3 ml of 50% ethanol and mix. Add 4.0 ml of methylene dichloride, stopper and shake vigorously for 10 sec. Let stand in the dark until the two layers separate (5 min). Remove 4 ml of the lower dichloromethane layer and transfer to an appropriate cuvette containing a couple of drops of ethanol. Measure the OD of the coloured product against a water blank at 520 nm. Readings should be taken within 30 min.

Calculation:

$$\text{mg Ketosteroid per day} = \frac{\text{OD of unknown}}{\text{OD of standard}} \times \frac{30}{1000} \times \frac{\text{total volume of urine}}{\text{urine aliquot used}}.$$

Note:

If the urine is kept refrigerated and if the analysis is to be carried out without much delay, no preservative need be added. For long storage, 5-10 ml conc. HCl may be added.

Reference:

Lipids and Steroid Hormones in Clinical Medicine. F. W. Sunderman and F. W. Sunderman Jr. (Eds.), J. B. Lippincott Co., Philadelphia (1965).

f. 17 - Hydroxycorticosteroids

Principle:

Total urinary 17-hydroxysteroids (free as well as conjugated) are extracted into n-butanol at an

acid pH. The steroids are then quantitated by the Porter-Silber reaction which involves formation of phenylhydrazones, with 17, 21-dihydroxy-20-ketosteroids in acid.

Reagents:

1. n-Butanol : If the blank values are very high the solvent should be purified in the following manner.

One l of butanol, add 100 ml of phenyl hydrazine-sulphuric acid reagent. Allow to stand for one week. Wash twice with 500 ml of distilled water. Place in the refrigerator for several hours. Decant butanol off the water layer. Add 30 g Na_2SO_4. Replace in the refrigerator overnight. Decant off supernatant and distil. Collect the fraction which boils at a constant temperature of 117 C.

Remove the water layer of the fraction boiling below 117°C. Treat the butanol layer with anhydrous Na_2CO_3, 30 g per litre. Allow to stand overnight and redistil collecting the fraction which boils at 117°C.

The OD should not exceed 0.025 when the colour reaction is carried out in 1 ml of the butanol plus 4 ml of phenylhydrazine-sulphuric acid reagent.

2. 25 and 50% H_2SO_4
3. Phenylhydrazine-sulphuric acid reagent : Recrystallise phenylhydrazine hydrochloride twice from ethanol. Dissolve 50 mg of this in 100 ml of 50% H_2SO_4.
4. Standard solution of hydrocortisone (Stock 200 μg/ml) : Dissolve 20 mg pure hydrocortisone in 100 ml n-butanol.

Working standard A (20 μg/ml) : Dilute 10 ml of stock to 100 ml with n-butanol.

Procedure:

Take 3 ml of urine in a 10 ml beaker. Acidify to pH 1.0 by dropwise addition of 25% H_2SO_4. Transfer completely to a 50 ml conical flask or glass stoppered test tube. Add 2.5-3 g of anhydrous ammonium sulphate. Add 3 ml of n-butanol and 30 ml of chloroform. Shake the tubes vigorously for 3 min. If conical flask is used, shake it slowly on a mechanical shaker for 20 min. Allow the two phases to separate. Discard the top aqueous phase. Filter the bottom solvent phase through Whatman No. 1 filter paper into another clean test tube. Pipette two 11 ml aliquots into separate glass stoppered test tubes.

Take 1.0 ml of working standard A in duplicate. Add 10 ml chloroform to each tube. Set up blanks in duplicate by taking 1 ml n-butanol and 10 ml chloroform. Mark one tube each as C and other as T. Add 4 ml 50% H_2SO_4 to C. Add 4 ml of phenylhydrazine-sulphuric acid reagent to T. Stopper the tubes and shake intensely for 15 sec. Allow them to stand for 15-20 min and transfer the upper acid layer into another test tube. Incubate the acid layer in a waterbath at 60°C for 45 min in dark. The developed colour is stable for atleast 20 hr. Read the OD in a spectrophotometer at a wavelength of 410 nm.

C series are read against H_2SO_4 blank and T series against phenylhydrazine-sulphuric acid blank. Take the readings first for C and then for T.

Calculation:

OD of standard (20 μg) = S, (OD for 20 μg should be double that for 10 μg).
OD of C = U_C
OD of T = U_T

$$\frac{U_T - U_C}{S} \times 20 \times \frac{33}{11} \times \frac{\text{total urinary volume (TV)}}{3 \text{ (vol. taken for analysis)}}$$

$$\text{mg 17-Hydroxysteroid/24 hr} = \frac{U_T - U_C}{S} \times \frac{20 \times TV}{1000}$$

Note:

Urine should be collected under glacial acetic acid and stored in cold.

References:

1. J. Clin. Endocrinol. Metab. **15**, 505 (1950).
2. Metabolism, **1**, 511 (1952).
3. Ibid. **3**, 489 (1954).
4. Ibid. **8**, 432 (1959).

g. Urinary pregnanediol

Principle:

Urine is subjected to mild acid hydrolysis to release pregnanediol from the conjugates. The free pregnanediol is extracted into a suitable solvent and after few purification steps the hormone is estimated spectrophotometrically using sulphuric acid as the colour reagent.

Reagents:

1. 1N NaOH
2. Standard solution of pregnanediol (100 μg/ml): Weigh exactly 1 mg pregnanediol and dissolve in 10 ml chloroform.

Procedure:

Filter 5 ml urine and add 15% by volume of conc. HCl. Dilute to 10 ml with water. Hydrolyse in a boiling water bath for 15 min. Cool in ice cold water. Extract with double the volume of hexane once and with equal volume of hexane, twice. Pool the hexane extracts and wash with 10 ml of 1 N NaOH. Discard the NaOH layer. Wash the extract with 10 ml of distilled water. Add Na_2SO_4 and let stand for 1 hr. This helps in removing moisture from the hexane extract. Evaporate the solvent by distillation. Dissolve the residue in 0,25 ml of chloroform.

TLC: Prepare 0.5 mm thick silica gel plates. Activate the plates before use at 110°C for 15 min. Apply the extract on the plate. Simultaneously run standards corresponding to 20, 40, 60, 80 and 100 μg. Run an ascending chromatography in the solvent system chloroform-acetone (9 : 1) for 45 min. Remove the plates and air dry them. Spray the plates with distilled water. The pregnanediol spots will be visible as greyish spots. Mark areas of grey spots corresponding to the standard pregnanediol and heat the plates at 110°C for 15 min. Scrape the marked areas and transfer the scrapings to the centrifuge tubes. Add 3 ml conc. H_2SO_4 to the tubes, and mix the contents thoroughly. Allow the tubes to stand for 15 min and then centrifuge. Take the supernatant and measure the OD at 430 nm using H_2SO_4 as blank.

Prepare a standard curve by plotting concentration of standard against OD and read the values in the test sample off it.

Calculation :

$$\text{mg Pregnanediol/24 hr} = \frac{\text{Concentration in test sample}}{\text{Volume of urine taken for analysis}} \times \frac{\text{Total urine volume}}{1000}.$$

Note :

1-4 mg can be estimated with fair accuracy. The recoveries range from 90 – 100%. Number of samples (8-10) can be analysed in one working day. Twenty four hour urine is collected without preservative and stored at 0°C till analysis.

Reference :

J. Chromat. **14**, 520 (1964).

3. OTHER HORMONES

a. Triiodothyronine - RIA

Principle :

Triiodothyronine (T_3) in plasma is measured by radioimmunoassay.

Reagents :

1. 50% V/V Propylene glycol.
2. 0.075 M Barbital buffer, pH 8.6 containing 0.1% BSA, 0.02% merthiolate and 1.5% 8 – anilino – 1 – naphthalene sulphonic acid.
3. ^{125}I – Triiodothyronine (sp. act. 30 μCi/μg) : Take about 0.5 μg and dilute with 50% propylene glycol to obtain a solution of 100 ng/ml. Make further appropriate dilutions with 50% propylene glycol. Prepare the working solution by diluting with barbital buffer to obtain a solution of 50 pg/0.1 ml.
4. Standard T_3 solutions : Weigh accurately 10 mg L – T_3 (free acid) and dissolve it in a few drops of 0.1 N NaOH. Add 1 ml 50% propylene glycol and make up the volume with water to 10 ml. Make further dilutions with barbital buffer to obtain a 10 ng/ml solution.

Working standards : Make further dilutions with barbital buffer to obtain solutions ranging from 0.25 – 10 ng/ml.

5. Anti - T_3 serum from rabbits.

Production of anti – T_3 serum in rabbits : Dissolve 50 mg BSA in 25 ml water. Filter the solution. Add 30 mg morpho CDi (1 – cyclohexyl – 3 – (2 - morpholinyl – 4 ethyl) carbodimide metho – p – toluene. Dissolve 20 mg T_3 in 5 ml dimethyl formamide. Add this to the above solution dropwise and with constant stirring. Keep the pH constant with the aid of dilute HCl and NaOH. After 10 min, add 10 mg morpho CDi. Keep the solution in dark, with constant stirring for 18 hr. Dialyse against running distilled water for 72 hr. Lyophilise and store in the cold.

Testing for effective conjugation can be done by taking the absorption spectrum of the product. Albumin has an absorption maximum at 280 nm and L – T_3 at 320 nm. The conjugate has absorption maximum at both wavelengths. The T_3 conjugate is mixed with complete Freund's adjuvant and

injected into rabbits (Refer Insulin-RIA). Establish the dilution required for 50% binding of radioactive T_3.

6. Anti – rabbit gamma globulin serum from guinea pigs.
7. Serum from normal rabbits (1 : 300).

Procedure :

Standard solutions of L – T_3 or test serum 0.1 ml, anti – T_3 antiserum 0.1 ml, barbital buffer 0.8 ml, mix, incubate for 24 hr at 4°C and then add ^{125}I – T_3 0.1 ml. Mix, incubate for 48 hr at 4 C and add normal rabbit serum 0.1 ml and precipitating serum 0.1 ml.

Mix and incubate for 20 hr at 4°C. Centrifuge the tubes at 3000 rpm in cold for 30 min. Transfer the supernatant into a separate test tube and count radioactivity in the supernatant as well as in the precipitate. Plot B/F ratio against the concentrations of T_3 and read values for test serum off the graph.

References :

1. J. Clin. Endocrinol. Metab. **33**, 509 (1971).
2. Ibid. **34**, 938 (1972).

b. Thyroxine - competitive protein binding assay

Principle :

Total thyroxine in serum is estimated by the competitive protein binding technique, principle of which is similar to that described for plasma cortisol assay. The free hormone is adsorbed on to an anionic resin.

Reagents :

1. 0 1 N NaOH
2. 1% Phenol
3. 50% V/V Propylene glycol
4. 0.75 M Barbital buffer, pH 8.6 : Dissolve 15.464 g of sodium barbitone in 800 ml distilled water (warm if necessary) and add 30 ml of 0.3 M HCl. Adjust the pH to 8.6 with dilute HCl and make up the volume to 1 l with water.
5. Anion exchange resin : Dowex 1 x 8 (200 - 400 mesh) chloride form. Soak the resin in barbital buffer overnight. Rinse the resin in buffer several times until the pH of the washed buffer is nearly 8.6. Dry the resin at room temperature.
6. ^{125}I – Thyroxine (specific activity 100 – 150 μCi/μ g): This is supplied in 50% propylene glycol. Dilute in 50% propylene glycol to obtain a working solution of 100 ng/ml and store in cold.
7. Standard thyroxine solution (Prepared from L – thyroxine, free acid), first stock solution (1 mg/ml) : Weigh accurately 1.0 mg of T_4 and add 1 ml propylene glycol. Add 0.1 N NaOH, 1 ml at a time with stirring until a clear solution is obtained. Make up the volume to 10 ml with distilled water. Mix and store at – 20°C. Stable for a year.

Second stock solution (10μg/ml) : Add 0.5 ml 0.1 N NaOH solution, 1 ml propylene glycol and 1 ml first stock solution to a 100 ml standard flask. Dilute to mark with water, mix and store in the refrigerator. The sloution should be prepared fresh every 2 weeks.

Working standard (100 ng/ml) : Mix 0.5 ml of 0.1 N NaOH and 1 ml second stock solution. Make up the volume to 100 ml with ethanol. Prepare the solution fresh every day.

8. TBG $^{125}I - T_4$ mixture : To 1 ml normal human serum, add 1 ml propylene glycol, 1 ml 1% phenol and $^{125}I - T_4$ solution. Make up to 100 ml with barbital buffer. Store in a brown bottle in the cold. It is preferable to prepare the solution 24 hr prior to the assay.

Procedure:

Take 0.2 ml of the test serum in a glass stoppered test tube. Add 0.4 ml ethanol and mix using a vortex mixer. Centrifuge at 2000 rpm for 10 min. Take 0.1 ml and 0.2 ml aliquots of the supernatant, evaporate to dryness in a water bath at 45°C under a gentle stream of commercial nitrogen. Take varying volumes of the working standard solution ranging from 0.02 - 0.2 ml in duplicate. Evaporate to dryness. Add 1 ml of the TBG - T_4 mixture to all the tubes and mix gently. Place the tubes in a metabolic shaker at 45°C for 8 min. Shake again to dissolve the dry residue. Immediately transfer to an ice bath and keep for 20 min. Add about 200 mg of the resin to each tube and shake gently for a min. Add 1.5 ml barbital buffer immediately and centrifuge for a while to help the resin to settle down. Decant the supernatant completely into another tube. Count the radioactivity in the supernatant and the resin in a well type counter.

Calculation :

Plot a graph with varying concentrations of T_4 versus either % binding or bound/free ratio. Read the concentration in the test sample off the graph and multiply by the correction factor (30 for 0.1 ml aliquot and 15 for 0.2 ml aliquot) to get the concentration per ml of serum.

References :

1. J. Clin. Endocrinol. Metab. **24**, 187 (1964).
2. J. Lab. Clin. Med. **66**, 161 (1965).

c. Protein bound iodine

Principle :

Serum hormonal iodide is precipitated by Somogy's zinc hydroxide precipitation technique. The precipitate is made alkaline, incinerated and the iodide in the ash is quantitated by the use of ceric arsenite system. Iodide acts as a catalyst in the following reaction

$$2Ce^{++++} + H_2O \longrightarrow 2Ce^{+++} + H_2AsO_4 + 2H^+.$$

The conversion of ceric ions to cerous ions is proportional to the amount of iodine present.

Reagents :

All the reagents should be prepared in deionised water or in glass distilled water.

1. 10% $ZnSO_4$
2. 0.5 N NaOH
3. 4N Na_2CO_3 : Dissolve 212 g of anhydrous sodium carbonate in 1 l water.
4. Arsenious acid reagent : Dissolve 0.988 g arsenious oxide (As_2O_3) in 10 ml of 0.5 N NaOH in a beaker with the aid of heat. Add this to approximately 800 ml water. Add next, 20 ml conc. HCl and 39.6 ml conc. H_2SO_4 slowly and mix continuously. Cool to room temperature and make up the volume to 1 l with distilled water.

5. Ceric ammonium sulphate: Take 600 ml water and add slowly 48.6 ml conc. H_2SO_4. Dissolve 20 g of ceric ammonium sulphate in this solution and cool. Make up the volume to 1 l with water.

6. Standard potassium iodide solution, first stock solution (100 μg/ml): Weigh very accurately 130.8 mg anhydrous KI and dissolve in 1 l of water. Store in the cold.

Second stock solution (4 μg/ml): Take 1 ml of the first stock iodide solution in a 25.0 ml volumetric flask and make up the volume with water.

Working standard solution: Take 1.0, 2.0, 3.0 and 4.0 ml fractions of the second stock solution and dilute each to 100 ml with water in a volumetric flask. These solutions will contain 0.04, 0.08, 0.12 and 0.16 μg iodide/ml respectively.

Procedure:

Take 0.1 ml serum or plasma in a 15 x 125 mm test tube. Add 6.0 ml water and 1.0 ml $ZnSO_4$ solution and mix with a glass rod. Add 1.0 ml 0.5 N NaOH, mix and let it stand for 10 min for complete precipitation of proteins. Centrifuge the tubes at 2000 rpm for 10 min. Wash the rod with 1 ml water into the tube. Discard the supernatant solution. Add 5.0 ml water to the protein precipitate and stir with a glass rod and centrifuge. Repeat the washing of precipitate 3 times. Add 1.0 ml 4N Na_2CO_3 to the precipitate and mix. Keep the tubes in a special aluminium or clay block and dry the contents of the tubes in a dry air oven at 90–100°C overnight.

See that the contents are absolutely dry. Place the tubes in a metal rack capable of withstanding a temperature of 600°C. Incinerate for 1 hr at exactly 600°C. Switch off the furnace and take out the tubes when the temperature comes down to 400°C. Usually ashing will not be complete in the first incineration and black specks of carbon sediment will be found. In such a situation cool the tubes and add 2–3 drops of water, dissolve the ash and dry it overnight at 90–100°C. Reincinerate the tubes for 20 min at 600°C. Remove the tubes when temperature reaches 400°C.

Take 1.0 ml of each working standard in duplicate. Add 1.0 ml Na_2CO_3 solution. Take 1.0 ml water instead of the iodide solution for the blank. Dry the contents of the tubes at 90-100°C overnight. The standard solution should not be incinerated.

Add 10.0 ml arsenious acid reagent to all the tubes. Let it stand for 15 min and centrifuge for 15 min at 2000 rpm. Take a 5.0 ml aliquot from each tube and keep in a water bath at 37°C for 15 min. Add 1.0 ml ceric ammonium sulphate to each tube at intervals of 1 min and incubate at 37°C exactly for 20 min. Read the colour in each tube exactly 20 min after the addition of the ceric ammonium sulphate solution. Take the readings in Klett-Summerson photometer using filter No. 42.

Calculation:

Prepare the standard curve by plotting log of absorbance against concentration of iodide. This can be easily done by using a semi-log paper. Read the value of the unknown sample from the standard curve. Express the values as μg iodide/100 ml plasma.

$$\mu\text{g PBI/100 ml plasma} = \mu\text{g iodide in unknown} \times \frac{10}{5} \times \frac{100}{1}$$

Note:

Either serum or plasma can be used for the estimation of PBI. Where plasma is to be used, the blood should be collected in heparinised containers.

All the reagents should be stored either in polythene or hard glass containers. The glassware should all be of hard glass and rinsed with glass distilled water before use.

References :

1. J. Clin. Invest. 30, 55 (1951).
2. Clinical Chemistry. Principles and Techniques, R. J. Henry (Ed.), Harper and Row, New York, p. 937, (1964).

d. ^{131}I Uptake by thyroid

Principle :

Iodine is selectively trapped and retained by the thyroid gland. This trapping mechanism is used as a diagnostic test by administering a tracer dose of radioactive iodine and determining the percentage of radioactivity retained by the thyroid at any given time.

Material and equipment :

1. Radioactive ^{131}I : ^{131}I is purchased from Bhabha Atomic Research Centre, Bombay. This is supplied as capsules, each containing 50 μCi.

2. Radioactive counter : A medical spectrometer (crystal scintillation counter supplied by Bhabha Atomic Research Centre, Bombay) is used for determining the thyroidal ^{13}I uptake. All measurements of radioactivity are made at a fixed distance from the probe.

Procedure :

Take the background count of the instrument for 10 min. Place the ^{13}I capsule in a 100 ml beaker and count the radioactivity for 10 min. Administer the capsule orally to the subject. The subject should be fasting overnight. Two and 24 hr later, the thyroidal uptake is determined as follows :

Adjust the probe vertically, over the thyroid of the subject. Count the radioactivity for 10 min. Position the probe over the left thigh, midway between the hip and the knee joints. Count the radioactivity for 2 min. This gives the extrathyroidal radioactivity.

Calculation :

Calculate the cpm for all measurements.

Net cpm in thyroid = cpm in thyroid – cpm over the thigh.

$$\text{Percent thyroidal } ^{131}I \text{ uptake} = \frac{\text{net radioactivity in thyroid} \times 100}{\text{radioactivity administered to the subject}}.$$

Reference :

Clinical Aspects of Iodine Metabolism, Blackwell Scientific Publications, Oxford, p. 208 (1964).

e. Vanilyl mandelic acid

(i) Ferricyanide method

Principle :

VMA (3 – methoxy–4 – hydroxy mandelic acid) is extracted from urine and oxidised to vanillin

by potassium ferricyanide at acid pH in the presence of zinc. The vanillin is estimated spectrophotometrically.

Reagents:

1. 0.5 M Phosphate buffer, pH 7.0: Dissolve 68.0 g of K_2HPO_4 in about 800 ml of water, adjust the pH to 7.0 and dilute to 1 l.
2. 4M Potassium phosphate: Dissolve 139. 3 g of K_2HPO_4 in 200 ml of water.
3. 0.6% Potassium ferricyanide: Keep refrigerated. Renew after 1 month.
4. 1.2% $ZnSO_4$
5. 0.5 N Ammonia solution containing 6% NaCl
6. Standard VMA: 100 μg/ml in 0.1 N HCl. Keep refrigerated. Renew after 3 months.

Procedure:

Take 50 ml of urine in a 250 ml conical flask. Add 5 ml of 10 N H_2SO_4 and 4 g florisil. Shake for 10 min and filter. Saturate 44 ml of the filtrate (40 ml of urine) with NaCl and extract first with 88 ml and then with 44 ml ethyl acetate by shaking for 10 min. Reextract the combined ethyl acetate extracts with 10 ml of phosphate buffer. Separate the aqueous layer. Wash the ethyl acetate layer with 2 ml of water. Measure the volume (P) of the aqueous extract including the washings. Transfer the aqueous extract to a small beaker and add slowly with stirring 5 ml concentrated HCl at 0°C. Measure out 4 aliquots (a, b, c and d) of 3 ml each. Set up the assay as follows:

	a	b	c	d
Standard solution (ml)	—	0.1	0.2	—
Zinc sulphate solution (ml)	0.6	0.6	0.6	0.6
Potassium ferricyanide (ml)	0.6	0.6	0.6	—
Distilled water (ml)	—	—	—	0.6

Incubate all tubes at 37°C for 2 hr. Protect from bright light. Cool and add 0.5 ml of 4 M K_2HPO_4 and adjust the pH to 7.0 with 5N NaOH. Add 5 volumes (about 25 ml) of toluene. Shake for 10 min. Add 4 ml of ammonia-NaCl reagent to the toluene extract. Shake for 5 min. Separate the aqueous layer which contains vanillin.

Measure the optical density of samples, a, b and c at 360 and 450 nm against sample d. Alternatively record absorption spectra between 300 and 450 nm.

Calculations:

$b - a = Rs_1 = 10\,\mu g$ standard
$c - a = Rs_2 = 20\,\mu g$ standard

Rs_2 should be nearly double ($\pm$ 15%) of Rs_1. Calculate value of VMA in a, $\frac{a \times 10}{RS_1} = U$

This is the value for the 3 ml aliquot taken from the total volume (TV).

$$\text{VMA } \mu g/24 \text{ hr} = \frac{U \times P}{3} \times \frac{TV}{40},$$

References:

1. Methods in Biochemical Analysis. Suppl. on Biogenic Amine, D. Glick (Ed.) Vol. 22 (1971).
2. Am. J. Clin. Path. 34, 293 (1960).

3. Clin. Chim. Acta. **7**, 285 (1962).

(ii) Periodate method

Principle :

VMA in urine is extracted selectively into ethyl acetate and oxidised to vanillin by periodate. The absorbance of vanillin is measured at 360 nm.

Reagents :

1. 6 N HCl
2. 1 M Potassium carbonate
3. 2% Sodium metaperiodate : Prepared freshly each week.
4. 10% Sodium metabisulphite : Keep in the refrigerator and prepare freshly each week.
5. 5N Acetic acid
6. 1 M Phosphate buffer, pH 7.4
7. Vanillin standard, 100 μg/ml in water

Procedure :

Collect a 24 hr specimen of urine into a bottle, containing 10 ml of 6 N HCl. Keep it cool. Measure a volume equal to 0.2% of the total specimen into a 50 ml glass stoppered tube, dilute to 5.5 ml with water and add a further 0.5 ml of 6 N HCl. Saturate with NaCl (about 3 g is required) and add 30 ml of ethyl acetate. Shake mechanically for 5 min to extract the phenolic acids. Centrifuge and transfer 25 ml of the ethyl acetate layer to a second glass stoppered tube and extract the phenolic acids with 1.5 ml of 1 M potassium carbonate by shaking for 30 min. Centrifuge again and transfer 1.0 ml of the carbonate layer to a third tube, add 0.1 ml of periodate solution and place the tube in a water bath at 50°C for 30 min to convert the hydroxy-methoxy-mandelic acid to vanillin. Cool the tube and add 0.1 ml of sodium metabisulphite solution to reduce the residual periodate. Then neutralise with 0.3 ml 5N acetic acid and 0.6 ml of 1 M phosphate buffer, pH 7.4. The pH can be checked at this point. It should be below 8.8 and be yellow when a drop of 0.04% aqueous cresol red is added. Shake for 3 min with 20 ml toluene to extract the vanillin. Centrifuge and transfer 15 ml of the toluene layer to a further 50 ml glass stoppered tube and extract the vanillin into 4 ml of 1 M potassium carbonate. Centrifuge and read at 360 nm against a reagent blank prepared by using water instead of urine.

Take 0.1 ml of the vanillin solution containing 10 μg and add 3.9 ml of 1 M potassium carbonate. Read as for the test.

Calculation :

$$\text{mg 4 - Hydroxy-3-methoxy-mandelic acid/24 hr urine} = \frac{\text{Reding of unknown}}{\text{Reading of standard}} \times \frac{10}{1000} \times \frac{100}{0.2} \times \frac{30}{25} \times \frac{1.5}{1.0} \times \frac{20}{15} \times 1.3 = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 15.6$$

The factor 1.3 is to convert vanillin to 4-hydroxy-3 - methoxy - mandelic acid. The recovery of added hydroxy-methoxy-mandelic acid is given as about 85 per cent.

Note :

For unire sample run a urine blank as described below:

For this proceed as above to the point at which 1.0 ml of carbonate layer is taken. Place this in the water bath without adding periodate. Then cool and add 0.1 ml of sodium metabisulphite before

adding 0.1 ml of periodate. Complete as described for the test. Subtract this blank reading from the total test reading.

Reference :

Clin. Chim. Acta. **7**, 285 (1962).

f. Serotonin

Principle :

The 5-hydroxy indoles are quite fluorescent in acid solutions or when they are coupled with ninhydrin. Since in the peripheral blood samples the interference by other 5 – hydroxy indoles are less, the fluorescence of serotonin can be straight away measured in an acid solution. In the case of brain where other 5-hydroxy indoles like 5-hydroxy tryptophan and 5 – hydroxy indole acetic acid may also contribute for the fluorescence, a better isolation and more specific condensation with ninhydrin is used.

Reagents :

1. Alumina : About 50 g of chromatographic grade alumina is boiled in 250 ml 1 N HCl for 30 min. Then wash with 20 x 100 ml of water or until the pH of the washings has risen to between 4 and 5. Finally, it is left to dry overnight at room temperature and then heated at 200°C for 2 hr.

2. n-Butanol : Batches of 500 ml n-butanol are washed successively with 50 ml of 1 N NaOH, 50 ml of 1N HCl and 4 x 50 ml of water in separating funnels and then saturated with NaCl.

3. Acid-butanol : One l of washed and salt saturated butanol is shaken with 0.85 ml concentrated (1.18 g/ml) HCl. Then 1.0 g potassium metabisulphite and 0.1 g of ethylene diamine tetraacetate disodium salt are added and the mixture shaken thoroughly.

4. 0.35 M Borate buffer, pH 10.0 : Boric acid (31.4 g) is dissolved in 1 l water and 55 ml of 10 N NaOH are added. This is then saturated with n-butanol and NaCl and adjusted to pH 10.0 if necessary.

5. 0.05 M Phosphate buffer, pH 7.0 : A mixture of 3.54 g of KH_2PO_4 and 5.77 g of Na_2HPO_4 is prepared and 0.233 g portions are weighed out for dissolving in 25.0 ml of water on the day of use.

6. 0.1 M Ninhydrin : Just before use, 0.036 g of ninhydrin is dissolved in 2.0 ml water (if stored frozen in a brown bottle, the ninhydrin reagent is stable for atleast 2 months and need not be prepared fresh for each experiment).

7. Protein precipitant : 10% $ZnSO_4$ and 1 N NaOH.

8. Standards; Platelet serotonin : Serotonin creatinine sulfate is used for the preparation of standards. The free base is 43.6% of the complex. Solutions of 100 μg/ml of serotonin free base are stable for several weeks when kept in cold. Working standard solutions should be freshly prepared.

Brain serotonin : 11.0 mg of serotonin creatinine sulfate equivalent to 5.0 mg of the free base is dissolved in 20 ml of 0.01 N HCl and stored in a deepfreeze. On the day of the experiment 0.5 ml of this solution is diluted to 25.0 ml with glass distilled water to a final concentration of 5.0 μg/ml.

Procedure :

Platelet isolation : About 10 ml of blood is collected in siliconised and heparinised glassware. All isolation procedure are carried out at 3°C using siliconised glasswares. The blood is centrifuged at 600 rpm for 45 min. The platelet rich plasma is transferred to another centrifuge tube and centrifuged

at 2000 rpm for 30 min. The plasma is decanted and the sedimented platelets are carefully suspended in 5 ml of cold saline to wash out adhering blood proteins. The tube is centrifuged at 2500 rpm for 20 min and the saline discarded. This washing procedure is repeated once more and then the platelets are suspended in about 3.5 ml of 0.02 N HCl. Gentle agitation causes lysing of the platelets.

Estimation : One ml from the lysate is removed for 5 HT assay. It is diluted to 2.0 ml with water and the proteins are precipitated by the addition of 0.2 ml of 10% $ZnSO_4$ and 0.1 ml of 1 N NaOH. After centrifugation, to the supernatant 0.3 ml of 12 N HCl is added and the fluorescence is measured with an activation at 295 nm and emission at 350 nm. Standards and reagent blank are treated in the same manner. A second aliquot of the lysed platelet solution is assayed for protein. Results are expressed as μg of serotonin per mg of platelet protein

Isolation of serotonin from brain tissue : The whole brain or portions of it as needed are homogenised in 10 ml of acid butanol reagent. Of this 4.5 ml is shaken for 2 min with 0.05 ml of standard serotonin solution containing 250 ng of the free base and then handled in parallel with remainder of this homogenate. All the homogenates are centrifuged at 1000 g for 5 min. Homogenisation, centrifugation and other procedures are carried out at 4°C.

A portion, 4.0 ml, of the supernatant butanol is then shaken with 2, 2, 4 - trimethylpentane (iso-octane) and 5 ml water for 2 min and centrifuged for 2 min at 1000 g. The upper organic phase is aspirated away and 4.5 ml of the water phase is added to 1.0 ml of 2 M sodium acetate solution and 0.2 g of alumina. The mixture is shaken for 5 min and then centrifuged for 5 min at 2000 g.

Then 5.0 ml of this supernatant fluid is transferred to a vessel containing 11.0 ml of butanol, 1.0 ml of borate buffer, pH 10 and 3 g of NaCl This mixture is shaken for 10 min and then centrifuged briefly. The butanol phase (10.0 ml) is shaken for 2 min with 15 ml iso-octane and 2.0 ml of 0.5 M phosphate buffer, pH 7.0. After centrifugation for 2 min at 1000 g the upper organic layer is removed by aspiration.

A portion (1.2 ml) of the aqueous phase is added to 0.1 ml of ninhydrin reagent and heated for 30 min at 75°C. An unextracted standard (0.01 ml standard + 1.12 ml, pH 7.0 buffer + 0.1 ml ninhydrin), a blank (1.2 ml, pH 7.0 buffer + 0.1 ml ninhydrin reagent) and a tissue blank (1.2 ml of mixed remains of the final tissue extracts, 0.1 ml of ninhydrin added just prior to reading the fluorescence) are heated simultaneously. After heating, the tubes are cooled and left to stand for atleast 1 hr at room temperature before reading the fluorescence at 495 nm, using activation at 385 nm. All wavelengths are uncorrected.

Calculations :

Platelet serotonin : A series of standards of serotonin 50-200 ng may be read in the fluorimeter and a concentration vs fluorescence graph may be drawn Then the concentration in the sample can be read against the fluorescence from the graph.

Brain serotonin : The amine content of each tissue sample is calculated by comparison with the internal standard. Of the 250 ng initially added to the homogenate (4/4.5) x 250 is extracted. This is represented by B-A, where A is the fluorescence units obtained from the final extract of the homogenate alone and B is the fluorescence units obtained from the portion to which standards has been added.

$$\text{Serotonin ng/g tissue} = \frac{\text{A–Blank}}{\text{B–A}} \times \frac{4}{4.5} \times 250 \times \frac{X}{Y},$$

where, Y is the weight of the tissue and X is the volume of acid butanol used for homogenisation.

Note :

For the isolation of platelet from blood, the sample should be drawn with siliconised syringes and the glassware should also be siliconised.

References :

1. Anal. Biochem. 23, 196 (1968).

2. Standard Methods of Clinical Chemistry, D. Seligson (Ed.) Academic Press Inc Publishers, New York and London. Vol. IV, p. 917 (1963).

g. Urinary catecholamines

Principle :

The only chemical method of sufficient sensitivity and specificity for the assay of catecholamine from normal urine is the trihydroxyindole (THI) - fluorimetric method. At slightly acidic pH values norepinephrine (NE) and epinephrine (E) are oxidised by iodine to form 2-iodoaminochromes. Ascorbic acid is added as a reducing agent to remove dissolved oxygen and thereby stabilise the compounds for the assay of fluorescence. The different estimation of NE and E is possible because both the amines are oxidised to the aminochrome stage at pH 6.5 but only E undergoes significant oxidation at pH 3.5.

Reagents :

1. 1.2 and 6 N HCl
2. Aluminium oxide (alumina) acid-washed, for chromatographic use. (See Serotonin for preparation).
3. 0.2 M EDTA : Dissolve (with heating) 37.2 g reagent grade disodium ethylene diamine tetraacetate ($Na_2C_{10}H_{14}O_8N_2.2H_2O$) in water, cool and make to 500 ml.
4. 0.5 and 5 N NaOH
5. 0.2 N Acetic acid : Dilute 5.8 ml glacial acetic acid to 500 ml.
6. 1 N Acetic acid : Dilute 28.8 ml glacial acetic acid to 500 ml.
7. 1 M Sodium acetate : Dissolve 68 g reagent grade sodium acetate ($CH_3COONa.3H_2O$) in water and dilute to 500 ml.
8. 1 M Acetate buffer, pH 6.5 : Adjust 400 ml of 1 M sodium acetate to pH 6.5 by the addition of approximately 6 ml of 1 M acetic acid.
9. 1 M Acetate buffer, pH 3.5 : Adjust 400 ml of 1 M acetic acid to pH 3.5 by the addition of approximately 33 ml of 1 M sodium acetate.
10. 0.1 N Iodine solution : Dissolve 0.635 g reagent grade iodine and 2.5 g reagent grade sodium iodide in 50 ml water.
11. 0.5 N Sodium thiosulfate : Dissolve 2.48 g reagnt grade sodium thiosulfate ($Na_2S_2O_3.5H_2O$) in water and make to 200 ml.
12. 1% Ascorbic acid : Dissolve 100 mg ascorbic acid in 10 ml deionised water just before use.
13. NaOH – ascorbic acid solution : Mix 7 volumes 5 N NaOH and 3 volumes 1% ascorbic acid immediately before use.
14. Stock standards, 1000 μgNE/ml : Dissolve 19.9 mg L-norepinephrine bitartrate monohydrate (MW. 337.2) in 10.0 ml 0.01 N HCl.

1000 μgE/ml : Dissolve 18.2 mg L-epinephrine bitartrate (MW. 333.3) in 10.0 ml 0.01 N HCl.

Working standards : Dilute 0.1 ml of each stock standard to 100 ml with 0.01 N HCl to give standard solutions of 1.00 μg/ml.

Procedure :

Preparation of the urine eluate : Filter an aliquot of urine equivalent to 10% of the 24 hour volume (50-250 ml) through two sheets of Whatman No. 1 filter paper on a suction funnel to remove any sediment. Transfer quantitatively to a 400 ml beaker, dilute smaller aliquots to approximately 200 ml with water, and add 5 ml 0.2 M EDTA and 3 g alumina.

With constant stirring on a magnetic stirrer and with constant monitoring using a glass electrode pH meter bring the mixture to pH 7-7.5 by the dropwise addition of 5 N NaOH. Adjust to pH 8.4 by the further dropwise addition of 0.5 N NaOH. Stir for 7 min adding 1-2 drops of 0.5 N NaOH as necessary to keep at pH 8.4 (to minimise the grinding of alumina by the stirring bar,elevate the beaker above the magnetic stirrer and run the strrer just fast enough to keep the alumina suspended). After 7 min of stirring rinse the electrodes and stirring bar with a few ml of deionised water. Allow the alumina to settle for 3-5 min and carefully decant and discard the clear supernatant. Transfer the alumina quantitatively with deionised water to a 1.2 cm diameter glass column. Allow the water to drain through and wash with two 10 ml portions of deionised water. Add 3 ml of 0.2 N acetic acid to the column and discard the effluent, this displaces water from the alumina. Add 9.4 ml of 0.2 N acetic acid (in two 4.7 ml portions) and collect the eluate in a calibrated centrifuge tube, it should be colourless. Dilute to 9.5 ml, add 0.5 ml of 0.2 M EDTA, centrifuge to remove occasional granules of alumina which may pass through and transfer to a test tube. The catecholamines are stable in this eluate for atleast several weeks at 2 C.

Prepare a series of test tubes with pH 6.5 buffer as follows :

Tube	Aliquot	Content
1.	0.20 ml NE working standard	NE external standard
2.	0.10 ml NE working standard	NE external standard
3.	0.20 ml E working standard	E external standard
4.	0.10 ml E working standard	E external standard
5.	—	Reagent blank
6.	0.20 ml of eluate	Sample tube
7.	0.20 ml of eluate	Sample blank
8.	0.10 ml eluate	Sample tube
9.	0.10 ml of eluate plus 0.10 μg NE working standard	Sample plus internal standard
10.	0.10 ml of eluate	Sample blank

To each tube add 1.0 ml of 1 N acetate buffer, pH 6.5. Add 0.10 ml of 0.1 N iodine solution to each tube and wait for 4 min. Add 0.50 ml of 0.05 N sodium thiosulfate to each tube to destroy the excess iodine. Mix the 5 N NaOH–1% asorbic acid solution and add 1.0 ml to each of the tubes except the sample blanks (tubes 7 and 10). To the latter two tubes add 0.70 ml of 5 N NaOH, wait for 15 min and then add 0.30 ml of 1% ascorbic acid to complete the blank. Dilute all the tubes to 5.0 ml with deionised water and allow them to stand in room light for an additional 30–45 min before reading.

Prepare a second series of tubes with pH 3.5 buffer exactly as described above, except use 0.10 μg E instead of NE in the internal standard tube (tube 9). Add 1.0 ml of 1 M acetate buffer pH 3.5 to each of the tubes, and treat them exactly the same as for the pH 6.5 series. At pH 3.5, E is oxidised quantitatively but NE remains almost unchanged.

Calculation:

For the determination of free catecholamines as NE equivalent, only the pH 6.5 figures are used. The catecholamine excretion in μg/day, is calculated as follows:

$$\frac{\text{Reading of 0.1 ml eluate at pH 6.5}}{\text{Reading of 0.1 } \mu\text{g NE internal standard}} \times 10 \times \frac{\text{urine volume}}{\text{ml aliquot analysed}}$$

For the differential estimation of NE and E the result must be calculated from simultaneous equations given below based upon readings obtained at the two pH values.

(a/b) NE + (c/d)E = reading of 0.1 ml eluate at pH 6.5
(e/b) NE + (f/d) E = reading of 0.1 ml eluate at pH 3.5

where, a = reading of 0.1 μg NE at pH 6.5 (internal standard)
b = concentration of NE standard (1 μg/ml)
c = reading of 0.1 μg E at pH 6.5 (external standard corrected for same degree of quenching as occurred for NE)
d = concentration of E standard (1 μg/ml)
e = reading of 0.1 μg NE at pH 3.5 (external standard)
f = reading of 0.1 μg NE at pH 3.5 (internal standard)
NE = concentration of NE in eluate in μg/ml
E = concentration of E in eluate in μg/ml.

Note:

A 24 hour urine specimen is collected in a glass bottle containing 15 ml of 6 N HCl (the final pH should be 3 or less). At this pH the catecholamines are stable in urine for atleast several days at room temperature and for years at 2°C.

All reagants are prepared with water which has been distilled and deionised. All reagents are stable at room temperature. Both stock and working standards are stable at 2°C for atleast 6 months. The EDTA is added to the urine and the eluate to prevent the formation of a gelatinous calcium-magnesium-phosphate precipitate.

If a spectrofluorimeter is available, set the instrument at 395 nm activation peak and 505 nm fluorescene peak for NE for the pH 6.5 series and activation peak at 410 nm and fluorescence peak at 520 nm for E for the pH 3.5 series. With filter fluorimeters use the 405 nm mercury line as the activating source and a secondary system which transmits in the yellow-green region. A satisfactory primary filter combination consists of Corning glass filters No. 4304, 3060 and 5970 (405 nm). The secondary filter system is composed of Corning glass filters No. 3385 and 4305, this passes light between 480 nm and 580 nm with peak transmittance at 505 nm.

For the pH 6.5 and 3.5 series adjust the instrument so that the galvanometer reading for the 0.20 μg NE and E standards respectively is about 80, and read the entire series of tubes. Record the reading for each tube, subtract the appropriate blank value, and calculate the average reading for 0.10 μg of each standard and 0.10 ml eluate at each pH. If any sample tubes read off the scale, the eluate should be diluted appropriately with water and reassayed.

Reference:

Standard Methods of Clinical Chemistry. D. Seligson (Ed.). Academic Press Inc Publishers. New York and London, Vol. III, p. 62. (1961).

IX. ENZYMES

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1. ACETYLCHOLINESTERASE

a. Colorimetric method

Principle:

Acetylcholinesterase (AChE) is a specific cholinestrase that catalyses the hydrolysis of a neurotransmitter acetylcholine.

$$\text{Acetylcholine} \xrightarrow{\text{AChE}} \text{Acetate} + \text{Choline}$$

The acetyl group reacts with alkaline hydroxylamine to form acetylhydroxamate. This acetylhydroxamate then reacts with ferric chloride in acid medium to form a coloured complex which can be read at 540 nm or using filter No. 54 of Klett-Summerson colorimeter.

Reagents:

1. 0.1 M Acetylcholine chloride : 181.66 mg/10 ml water. (Preserve frozen at-20 C).
2. 1 M NaCl : 5.845 g/100 ml.
3. 1 M $MgCl_2$: 20.33 g/100 ml water.
4. 0.5 M Tris-HCl, pH 7.5.
5. 0.2 M EDTA: Mix 7.44 g of EDTA in 50 ml water and add about 16 ml of 1 N NaOH to bring to neutral pH. Make upto 100 ml with water. Dilute 1 in 100 to get 2 mM solution.
6. Cocktail : Mix the following solutions (2-5). 1.0 M NaCl 13 ml, 1.0 M $MgCl_2$ 2.0 ml, 0.5 M Tris-HCl 10 ml and 0.2 M EDTA 10 ml.
7. 2 M Hydroxylamine-HCl : Dissolve 27.8 g in 200 ml water.
8. 3.5 M NaOH : Dissolve 28 g in 200 ml water.
9. Mix 7 and 8 just before use in 1:1 (V/V).
10. 6 N HCl.
11. 0.37 M Ferric chloride in 0.1 M HCl : 10 g in 100 ml of 0.1 M HCl. (Store in brown bottle).
12. Reaction medium : Mix the following solutions. Cocktail 17.5 ml, 0.1 M acetylcholine 2.0 ml and glass distilled water 5.5 (This reaction medium gives in final concentration – NaCl 130 mM, $MgCl_2$ 20 mM. Tris-HCl pH 7.5, 50 mM, EDTA, 0.2 mM and acetylcholine 4 mM).

Procedure:

Take 0.5 ml of reaction medium and 0.1 ml of (approximate, but to be decided by the experimenter) either 1:10 rat or human brain homogenate or 1 : 10 RBC haemolysate. Then add 0.4 ml water. Incubate for 30 min at 37°C.

Terminate the reaction by adding 2.0 ml of reagent 9 prepared freshly. Mix on vortex mixer. After 1 min, add 1 ml of reagent 10. Mix and add 1 ml of reagent 11 (acidic ferric chloride). Read using filter No. 54 in Klett or at 540 nm in Coleman.

Note :

It is, however, possible to improve the sensitivity of the method by cutting all the volumes to a fourth or fifth.

Failure to attain a high enough pH (about 11) after the addition of alkaline hydroxylamine or low enough pH (1.2-1.5) after the addition of HCl could result either in the appearance of precipitate or a colour. In such a case appropriate adjustments for HCl dilution become necessary.

b. Spectrophotometric method

Principle :

Thio analogue of acetylcholine is used as a substrate and the SH groups released are titrated with 5,5'-dithiobis-(2-nitro benzoic acid)-DTNB. The change in optical density can be followed either spectrophotometrically at 412 nm or even in any colorimeter using filter No. 42.

Reagents:

1. Cocktail number six from the colorimetric assay.
2. 0.1 M Acetylthiocholine chloride: 20.17 mg/ml of glass distilled water. To be preserved frozen in amber coloured bottle.
3. 1 mM DTNB : Dissolve 0.396 mg/ml in glass distilled water.
4. Reaction mixture: Mix the following. Cocktail 10.5 ml, 1 mM DTNB 3.0 ml (to be added fresh) and water 6.5 ml.

Procedure :

About 20-50 μg of rat brain homogenate or 10 μl of 1 : 10 erythrocyte suspension or 20-50 μg of rat or human erythrocyte membrane preparation give linear rate of reaction up to 10-15 min.

Take 2.0 ml of reaction mixture, 0.1 M acetylthiocholine 0.03 ml, enzyme preparation 0.10 ml and water 0.87 ml (Volume of enzyme preparation could be varied according to convenience).

The reaction is initiated either by the addition of enzyme or substrate.

A substrate blank without enzyme is necessary since acetylthiocholine always has 2–5% of free-SH groups (depending on the batch number and make) and also it is unstable.

Calculation :

Molar extinction coefficent of-SH group of DTNB is 14.3×10^3 at 412 nm.

For a 3.0 ml reaction volume it is $\frac{14.3}{3} = 4.43$.

$$\therefore \quad \mu \text{ moles/min} = \frac{\triangle \text{ OD per min}}{4.43}.$$

This when divided by protein will give specific activity.

Note:

The method is most suited for all types of kinetic studies.

References :

1. J. Biol. Chem. 180, 249 (1949).
2. Biochem. Pharmacol. 7, 88 (1961).

2. ALANINE AMINOTRANSFERASE

Principle :

Alanine aminotransferase catalyses the reaction

$$\text{L-Alanine} + \text{2-Oxoglutarate} \overset{\text{GPT}}{\rightleftharpoons} \text{Pyruvate} + \text{L-Glutamate.}$$

The product pyruvate formed oxidises NADH in the coupled enzymatic reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + \text{NAD}^+$$

This reaction is followed spectrophotometrically by measuring the disappearance of absorbance due to NADH at 340 nm with time.

Reagents :

1. 0.1 M Phosphate-1.0 M alanine buffer , pH 7.4 : Dissolve 0.2 g KH_2PO_4, 1.5 g K_2HPO_4 and 8.90 g L-alanine in distilled water, adjust pH to 7.4 and make up to 100 ml. Layer with 0.3 ml chloroform to prevent bacterial growth on storage.

2. 13 mM NADH solution : Dissolve 16 mg NADH-Na_2 and 15 mg $NaHCO_3$ in 1.5 ml distilled water.

3. Lactate dehydrogenase (0.5 mg protein/ml) : Dilute commercial LDH solution in glycerol with 50% (V/V) glycerol.

4. 0.66 M 2-Oxoglutarate : Dissolve 290 mg 2-oxoglutaric acid in approximately 2 ml distilled water, adjust pH with 1.0 N NaOH in an ice bath and make up to 3 ml with water (disodium salt 375 mg may be directly dissolved in 3 ml distilled water).

Procedure :

Prepare a reagent mixture by mixing phosphate-alanine solution, 30 volume, NADH solution, 0.5 volume and LDH solution 0.5 volume (stable for 12 hr at 25°C) and incubate at 25°C. Pipette into the cuvettes 3.1 ml reagent mixture and 0.5 ml serum sample, mix and incubate for 5 min. Add 100 μl 2-oxoglutarate solution, mix and follow the reaction (at 25°C) at 340 nm for 5-10 min.

Calculation :

Alanine aminotransferase activity (U/l) $= 1190 \times \triangle A_{340nm}/\text{min.}$

Note:

Store all reagents refrigerated at 0 – 4°C. Prepare reagents 2 and 4 freshly every 4 weeks.

Reference :

Methods of Enzymatic Analysis, H.U. Bergmeyer (Ed.), Verlag/Academic Press. Vol. II, p, 762 (1971).

3. ALKALINE PHOSPHATASE

a. Bodansky's method

Principle :

The method of Bodansky, modified by using β-glycerophosphate as the substrate, is used. The inorganic phosphate liberated from the substrate is estimated by Fiske and Subba Row method.

Reagents:

1. Substrate: Into a 100 ml flask are introduced in the following order, 3.0 ml of petroleum ether, 80.0 ml of distilled water, 0.5 g of sodium β-glycerophosphate, 0.424 g of sodium diethylbarbiturate and finally water to make up the volume. The solution is adjusted to pH 8.5 and stored in the cold.
2. 30% TCA
3. Refer phosphate estimation under Minerals.

Procedure:

Substrate solution 0.9 ml is measured into a centrifuge tube and placed in an incubator at 37°C until the solution reaches the incubator temperature. Serum 0.1 ml is added to it and mixed. At the end of the incubation period of 1 hr, 0.2 ml of 30% TCA is added to stop the reaction. The inorganic phosphate liberated by the enzyme action, is estimated in the filtrate. A blank tube is run with serum in which reaction is stopped at zero min.

Calculation:

Alkaline phosphatase activity is expressed in terms of mg inorganic phosphate liberated/hr/100 ml serum at pH 8.5.

References:

1. J. Biol. Chem. **99**, 197 (1932).
2. J. Biol. Chem. **66**, 375 (1925).

b. King and Armstrong method

Principle:

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{Alk. phosphatase}} \text{Nitrophenol} + H_3PO_4.$$

The amount of p-nitrophenol liberated in unit time, as determined in alkaline pH at 400-420 nm is a measure of the phosphatase activity.

Reagents:

1. 0.1 M Diethanolamine-1.25 mM p-nitrophenyl phosphate buffer: Dissolve 1.052 g diethanolamine, 8 ml 0.1 N HCl and 46.4 mg p-nitrophenyl phosphate in 85 ml double distilled water, adjust pH to 9.8 and dilute to 100 ml with distilled water. (Stable for a week at 0-4°C).
2. Sample: Fresh, nonhaemolysed serum is to be used.
3. 0.05 N NaOH

Procedure:

Pipette into tubes buffer-substrate solution (pH 9.8), 2.0 ml and 50 µl serum. Mix and incubate for exactly 30 min at 25°C. Stop the reaction by adding 10 ml 0.05 N NaOH. For blanks, the sample is added after the NaOH addition. Read the absorbance at 405 nm against the respective blanks.

Calculation:

$$\text{Alakline phosphatase activity (U/l)} = \frac{\triangle A_{405}}{30} \times \frac{12.05 \times 1000}{18.5 \times 0.05} = \triangle A_{405} \times 434.$$

Reference :

Methods of Enzymatic Analysis, H.U. Bergmeyer (Ed.), Verlag/Academic Press, Vol. II, p. 856 (1971).

4. D-AMINO ACID OXIDASE

Principle :

The amount of ketoacid (pyruvic acid) formed by liver homogenate after incubation with DL-alanine is measured.

Reagents:

1. 0 04 M DL-Alanine
2. 0.04 M Sodium arsenite prepared in 0.11 M NaCl
3. Krebs - Ringer solution : Refer Appendix (from which calcium is omitted)
4. 0.02 M Phosphate buffer, pH 7.4
5. 20% TCA
6. 2,4-Dinitrophenylhydrazine half saturated in 1N HCl
7. 2 N NaOH

Procedure :

In 100 ml conical flask the following reagents are taken ; 0.5 ml of 0.04 M sodium arsenite, 0.5 ml of 0.04 M DL-alanine, 2.0 ml of Krebs-Ringer solution (calcium free) and 1.0 ml of liver homogenate.

A flask containing all the reagents except DL-alanine (replaced by an equal quantity of the Ringer's solution) is also set up at the same time to serve as the sample blank. The air phase above the mixture in the flask is replaced by oxygen and the flasks incubated at 37°C for exactly 6 hr. Oxygen at low pressure is passed through the period of incubation. At the end of this, the reaction is stopped by adding 1 ml of 20% TCA. 5 ml of water is then added, mixed and filtered through Whatman No. 1 filter paper.

To 5 ml of the filtrate, 1 ml of 2,4-dinitrophenylhydrazine is added. After 10 min, 10 ml of 2 N NaOH is added and the solution diluted to 25 ml with water and read in the Klett-Summerson photoelectric colorimeter using filter No. 54. The blank value is subtracted and the amount of ketoacid (pyruvic acid) formed is read from a curve drawn with pure Na-pyruvate solution.

Nitrogen content of the liver is determined by macro-Kjeldahl method.

Calculation :

The enzyme content is expressed in terms of μ moles of pyruvic acid formed per g protein or per g liver (wet weight).

Reference :

J. Biol. Chem. **176**, 1371 (1948).

5. AMINOPYRINE N-DEMETHYLASE

Principle :

$$\text{Aminopyrine} \xrightarrow{\text{NADPH, } Mg^{2+} (O)} \text{4-Monomethylamino antipyrine} + \text{HCHO} \xrightarrow{\text{NADPH, } Mg^{2+}} \text{4-aminoantipyrine} + \text{HCHO}$$

Aminopyrine is a model substrate to study N-dealkylation of drugs and elicits a type I spectrum on binding to Cyt.P-450. The reaction rate may be followed by measuring either the formation of the dealkylated product, 4-aminoantipyrine or the quantity of formaldehyde formed.

Reagents:

1. 150 mM Potassium phosphate buffer, pH 7.4.
2. 75 mM Magnesium chloride.
3. 5 mM NADP.
4. 120 mM Sodium isocitrate.
5. Isocitrate dehydrogenase (ICDH) in 50% glycerol : 4.4 U/mg protein.
6. 120 mM Aminopyrine.
7. 75 mM Semicarbazide hydrochloride.
8. 10% $ZnSO_4$.
9. Saturated solution of $Ba(OH)_2$.

Procedure:

Take in 10 ml Erlenmeyer flasks the enzyme, cofactor and the substrate as indicated below (the last column indicates the final concentration of the constituents in the assay mixture).

Phosphate buffer	0.5 ml	50 mM
$MgCl_2$	0.1 ml	5 mM
NADP	0.1 ml	0.33 mM
Sodium isocitrate	0.1 ml	8.0 mM
ICDH (Sigma type IV)	10 μl	10 μg
Aminopyrine (or water for tissue blank)	0.1 ml	8.0 mM
Semicarbazide	0.1 ml	5.0 mM

Incubate the assay mixture for 15 min at 37°C in a Dubnoff shaker to ensure reduction of all the NADP to NADPH. Then add the liver microsomal suspension, 0.5 ml (containing 1.5 mg microsomal protein) and incubate for 10 min at 37°C with shaking (120 cycles/min). Stop the reaction after 10 min by adding 0.5 ml of 10% $ZnSO_4$ solution followed by 0.5 ml of saturated $Ba(OH)_2$ solution. Mix and transfer the contents to 5 ml tubes and centrifuge. Estimate the formaldehyde formed using 1 ml of the clear supernatant. A tissue blank, replacing substrate by water, must always be employed to correct for endogenous formaldehyde formation.

Estimation of formaldehyde:

Principle:

The formaldehyde trapped as the semicarbazone is measured by the spectrophotometric method based on the Hantsch reaction. In the Hantsch reaction the formaldehyde is condensed with ammonia and acetyl acetone to give the yellow coloured product, 3, 5,-diacetyl-1, 4-dihydrolutidine (DDL) which can be measured spectrophotometrically.

Reagent:

Nash reagent: To 30 g ammonium acetate, add 0.4 ml distilled acetyl acetone and make upto 100 ml with distilled water. When freshly prepared it is colourless and gives low blank values (on keeping the reagent turns yellow and gives higher blanks).

Procedure :

To 1.0 ml supernatant (sample) or water/tissue blank, add 0.5 ml of the Nash reagent and mix. Place the tubes in a water bath at 60°C for 30 min for colour development. Read the absorbances of the tissue blank and the samples (after centrifugation, if slightly turbid) against the water blank at 412 nm. The amount of formaldehyde formed may then be calculated after subtracting the tissue blanks and using the molar extinction coefficient of 8000.

Note :

The storage of liver homogenate at 0°C leads to losses in aminopyrine demethylase activity within a few hours. Also, demethylation of aminopyrine and codeine decreases rapidly even if the 10,000 x g supernatant is stored in an ice bucket. Microsomes must therefore be isolated without delay and these enzymes assyed as soon as possible.

The rate of the reaction proceeds linearly only for a short time (around 10 min) and therefore longer incubation periods must be avoided.

The condensation product of the Hantsch reaction is relatively stable but is affected by prolonged exposure to light and oxidising agents.

Reference :

Fundamentals in Drug Metabolism and Drug Disposition. B. N. La Du, H. G. Mandel and E. L. Way (Eds.), Williams and Wilkins Co., p. 546 (1971).

6. AMYLASE

Principle :

The activity is measured by determining the amount of starch it can split in a given time. The amount of starch in the reaction mixture is estimated by measuring the colour it produces with a weak iodine solution.

Reagents :

1. Standard starch solution : 0.8 g starch and 0.1 g benzoic acid are dissolved in boiling water, cooled and made upto 100 ml.
2. 0.1 M Phosphate buffer, pH 7.4 : 19 ml of 0.2 M NaH_2PO_4 and 81 ml of 0.2 M Na_2HPO_4 are mixed and diluted to 200 ml with distilled water.
3. Gram Lugol's iodine solution : 1.0 g iodine and 2.0 g KI are dissolved in distilled water and made upto 100 ml.
4. 5% H_2SO_4.

Procedure :

To 0.5 ml of starch solution in a large test tube is added 0.5 ml of phosphate buffer. To this is added 0.2 ml serum and the mixture incubated at 37°C for 30 min. At the end of this period 2 ml of 5% H_2SO_4 and 1 ml of iodine solution are added and the volume made upto 50 ml with distilled water. A control tube is prepared by adding 5% H_2SO_4 immediately after the addition of serum and followed similarly as for test. The colour is measured in a colorimeter using a red filter (640 nm) against the reagent blank without either starch or serum.

Calculation :

$$\frac{\text{OD control-OD sample}}{\text{OD control}} \times 4 \times \frac{10}{0.2} \text{ Units}$$

1 Unit = the amount of starch in mg hydrolysed by 10 ml of serum in 20 min.

Reference :

Am. J. Clin. Path. **27**, 714 (1957).

7. ANILINE HYDROXYLASE

Principle :

Hydroxylation of aniline is followed by determining the rate of formation of the product p – aminophenol according to the reaction :

$$\text{Aniline} \xrightarrow[Mg^{+}]{\text{NADPH (O)}} \text{p – Aminophenol}$$

Reagents :

1. 150 mM Potassium phosphate buffer, pH 7.4
2. 75 mM $MgCl_2$
3. 5 mM NADP
4. 120 mM Sodium isocitrate
5. 4.4 U/mg Protein isocitrate dehydrogenase (ICDH) in 50% glycerol solution
6. 20% TCA
7. 120 mM Aniline hydrochloride, (use only recrystallised aniline HCl).

Procedure :

Take in 10 ml Erlenmeyer flasks the following solutions (the last column gives the concentrations in the assay mixture).

Phosphate buffer, 150 mM	0.5 ml	50 mM
$MgCl_2$, 75 mM	0.1 ml	5 mM
NADP, 5 mM	0.1 ml	0.33 mM
Sodium isocitrate, 120 mM	0.1 ml	8 mM
ICDH	10 µl	10 U
Aniline HCl, 120 mM	0.1 ml	8 mM
Distilled water	0.1 ml	—

Incubate at 37°C for 15 min to convert all of the NADP to NADPH. Add the microsomal suspension, 0.5 ml (containing around 1.5 mg protein) to start the reaction and incubate at 37°C with shaking (120 cycles/min). At the end of 15 or 20 min stop the reaction by adding 0.5 ml of 20% TCA. Mix and transfer the contents to 5 ml tubes and centrifuge. Using 1.0 ml of the supernatant, estimate the p – aminophenol formed.

Estimation of p - aminophenol:

Principle:

p - Aminophenol reacts with phenol in alkaline medium to from the blue coloured phenol-indophenol complex which can be measured at 630 nm.

Reagents:

1. 10% Na_2CO_3
2. 2% Alkaline phenol in 0.2 N NaOH
3. p - Aminophenol standard in 6.67% TCA (0.2 – 2.0 μg/ml)

Procedure:

To 1.0 ml of the TCA supernatant, add 0.5 ml of 10% Na_2CO_3 solution and mix. Then add 1.0 ml of 2% phenol in 0.2 N NaOH, mix and place it in an incubator at 37°C for 30 min for development of the colour. Read the absorbance in a spectrophotometer at 630 nm. The amount of p – aminophenol may then be calculated by comparing with standards or using the millimolar extinction coefficient of the indophenol dye in TCA (E 630 = 28.4 cm^{-1} nm^{-1}).

References:

1. Fundamentals in Drug Metabolism and Drug Disposition, B. N. La Du, H. G. Mandel, and E. L. Way (Eds.), Williams and Wilkins Co., p. 569 (1971).
2. Toxicol. Appl. Pharmacol. **22**, 50 (1972).

8. ASPARTATE AMINOTRANSFERASE

a. Colorimetric method

Principle:

The enzyme catalyses the following reaction:

$$\text{Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AsAT}} \text{Oxaloacetate} + \text{Glutamate}$$

In the colorimetric procedure the oxaloacetate formed is converted to pyruvate and measured colorimetrically. Pyridoxal-5-phosphate is the coenzyme.

Reagents:

1. Buffered substrate: Dissolve 3.61 g DL-aspartic acid, 2.00 g KH_2PO_4 and 0.6 g α-ketoglutaric acid in about 80 ml glass distilled water. Adjust the pH to 7.4 with 3 N KOH and make up the volume to 100 ml with glass distilled water. Store in refrigerator, stable for 1 month.
2. Pyridoxal-5'-phosphate solution (PLP): Dissolve 2 mg of PLP in 4 ml distilled water. Prepare fresh and keep away from bright light.
3. 100% TCA (W/V) in glass distilled water.
4. Aniline-citrate solution: Dissolve 2 g citric acid in 2 ml glass distilled water and add 2 ml of freshly distilled aniline. Prepare fresh.

5. DNP : Dissolve 100 mg of 2, 4-dinitro phenylhydrazine (DNP) in 20 ml concentrated HCl and make the volume to 100 ml with glass distilled water.

6. Alcoholic potassium hydroxide : Dissolve 2.5 g KOH pellets in 2 ml glass distilled water and make up the volume to 100 ml with distilled alcohol.

7. Pyruvate standard stock solution : Dissolve 25 mg sodium pyruvate (20 mg pyruvic acid) in about 80 ml glass distilled water and make up the volume to 100 ml.

Procedure :

Add 9.8 ml glass distilled water to 0.2 ml of RBC (washed and stored) and keep for 1 hr.

Carry out duplicate analysis with all samples Take 0.5 ml of haemolysate in three sets of tubes (blank, basal activity and stimulated activity). Add 0.1 ml of PLP solution to one set of tubes (stimulated activity) and incubate all the tubes at 37°C for 30 min in a shaker water bath shaking at low speed.

Add glass distilled water to all the tubes to make the volume to 1 ml. Add two drops of 100% TCA solution to blank tubes. Add 0.5 ml buffered substrate solution to all the tubes. Incubate all the tubes at 37°C for exactly 30 min. Add two drops of 100% TCA to all the tubes except the blank tubes. Add 2 drops of aniline citrate to all the tubes. Shake well and leave at room temperature for 20 min. Add 0.5 ml DNP solution. Mix well and leave at room temperature for 5 min. Add 2 ml toluene, shake well and centrifuge at 2000 rpm for 5 min.

Transfer 1 ml toluene layer to clean dry tubes. Add 3 ml of alcoholic KOH and mix thoroughly. Measure OD at 510 nm against a blank containing 1 ml toluene and 3 ml alcoholic KOH solution.

Run a set of pyruvate standards (10-100 μg) similarly. Plot the absorbance against the concentrations.

Calculation :

Subtract the blank reading from the test and obtain the pyruvate content in each tube from the standard graph.

$$\text{Enzyme activity} = \frac{\mu\text{g pyruvic acid}}{88.07} \times \frac{10}{0.5} \times \frac{1}{0.2} \times \frac{60}{30}\ \mu \text{ moles pyruvate/ml RBC/hr.}$$

$$\text{Activation coefficient} = \frac{\text{Stimulated activity}}{\text{Basal activity}}.$$

Note :

In pyridoxine deficiency, the activity of AsAT is reduced but can be stimulated by the *in vitro* addition of pyridoxal-5' - phosphate. Extent of activation expressed as activation coefficient gives a measure of pyridoxine deficiency.

References :

1. Am. J. Clin. Nutr. **26**, 1101. (1956).
2. Ibid. **27**, 235 (1957).

b. Spectrophotometric method

Principle :

Erythrocyte aspartate aminotransferase (EAsAT) activity is assayed based upon the transamination of aspartic acid to 2-oxoglutaric acid. The resulting oxaloacetate is enzymatically transformed to malate by the NADH-dependent malate dehydrogenase (MDH). The consumption of NADH, ascertained by measuring the decrease in absorbance at 340 nm corresponds to the amount of oxaloacetate present which, in turn, reflects the degree of enzymatic activity. Vitamin B_6 in the form of pyridoxal-5-phosphate is the coenzyme.

1. Aspartate + 2-Oxoglutarate $\overset{\text{AsAT}}{\rightleftharpoons}$ Oxaloacetate + Glutamate.

2. Oxaloacetate + NADH + H^+ $\overset{\text{MDH}}{\rightleftharpoons}$ Malate + NAD^+.

Reagents:

1. Tris – aspartate buffer, 0.25 M aspartate in tris buffer; pH 7.6: Dissolve 12.11 g of tris in 150 ml of water. Add 400 ml of 0.1 M HCl. Dilute to 1 l. The resultant solution should be at pH 7.6 but small adjustment may be necessary. Dissolve 4.24 g of potassium aspartate in 100 ml of the buffer and adjust to pH 7.6 at 25°C. Store at 4°C. Stable for 1 month.

2. 0.2 M 2-Oxoglutarate solution : Dissolve 10.13 g 2-oxoglutarate sodium salt in 100 ml distilled water. Adjust pH to 7.6 if necessary using NaOH. Store at 4°C. Stable for 2 weeks.

3. 0.006 M NADH solution : Dissolve 5 mg of NADH in 1.0 ml of tris-aspartate buffer. Prepare freshly.

4. Malic dehydrogenase : Dilute the 10,000 units/ml to 2,000 units/ml with ammonium sulphate dilution solution provided by Sigma. Prepare freshly in 1 ml amounts.

5. 2.43 mM Pyridoxal-5-phosphate: 60 mg dissolved in 100 ml of 0.005 M NaOH. Stable in the refrigerator for one month. Working solution is prepared freshly. Dilute 1 ml of the above solution with 3 ml of distilled water.

Procedure :

Solution	Tube I (ml)	Tube II (ml)
Distilled water	2.40	2.10
Tris – aspartate buffer	0.50	0.50
PLP	—	0.30
MDH	0.05	0.05
NADH	0.05	0.05
Haemolysate	0.10	0.10

Mix the contents of the tube and place in water bath at 37°C for 30 min. Transfer the contents to a cuvette, start the reaction by the addition of 0.1 ml of 2-oxogultarate solution. Measure the change in absorbance at 37°C. The $\triangle$ OD is calculated between 3 min and 9 min.

Calculation :

One unit of EAsAT is expressed as μ moles of NADH oxidised/hr/ml of red cells. $\triangle$ A is the decrease of absorbance per min at 340 nm. The molar extinction of NADH at 340 nm is 6.22×10^3.

$$\text{EAsAT activity} = \frac{\triangle A \times 3.02 \times 60 \times 20}{6.22 \times 0.2}$$

$$= \triangle A \times 6174 \text{ U/ml.}$$

Results should be expressed as basal and stimulated EAsAT activity. The activity coefficient is the ratio of stimulated to basal activity.

References :

1. J. Clin. Invest. **34**, 131 (1955).
2. Clin. Chim. Acta. **17**, 353 (1967).
3. Am. J. Clin. Nutr. **15**, 67 (1964).
4. Int. J. Vit. Nutr. Res. **41**, 240 (1971).

9. BENZO (a) PYRENE HYDROXYLASE

a. Nebert and Gelboin method

Principle :

Benzopyrene is converted to phenols, arene oxides, dihydrodiols and quinones by the microsomal mixed function oxidases. The remarkable similarity of the fluorescent spectrum of an extract of the reaction products in alkaline pH to that of standard 3-hydroxybenzopyrne (3-HOBP) forms the basis of the spectrophotofluorimetric assay of benzopyrene hydroxylase (BPH).

Reagents :

1. 0.2 M Potassium phosphate buffer, pH 7.5
2. 30 mM Magnesium chloride
3. 1 mM EDTA - sodium salt
4. 4 mM NADPH - tetrasodium salt, type I (Sigma)
5. 2 mM Benzo (a) pyrene (Sigma) in dimethylsulfoxide
6. 1.0 N NaOH
7. 3 - Hydroxybenzo (a) pyrene (3-HOBP)
8. 1 mM Quinine sulfate, in 0.1 N H_2SO_4

Procedure :

Carry out all the assay in subdued light, in 10 ml Erlenmeyer flasks in a total volume of 1.0 ml. (The last column indicates the final concentrations of the constituents in the assay mixture).

Phosphate buffer 0.2 M, pH 7.5	0.5 ml	100 mM
EDTA, 1 mM	0.1 ml	0.1 mM
$MgCl_2$, 30 mM	0.1 ml	3.0 mM
NADPH, 4 mM	0.1 ml	0.4 mM
Benzo (a) pyrene, 2 mM	40 μl	80 μM
Microsomal suspension	30-60 μ l	100 μg protein
Water to make up the final volume to 1.0 ml.		

After preincubation for 1 min the reaction is started by the addition of substrate. Incubate for 10 min at 37 C with shaking (120 cycles/min). Stop the reaction by adding 1.0 ml of chilled acetone. Determine the enzyme activities in duplicate and compare with a blank where acetone has been added prior to incubation.

Extract the metabolites into 3.25 ml of hexane by incubating at 37°C for 10 min with shaking. Transfer the contents to a centrifuge tube and centrifuge. Pipette 2.0 ml from the 3.30 ml of the organic phase into another tube and extract the alkali soluble metabolites into 1.0 ml of 1.0 N NaOH by vortex mixing for 1 min. Centrifuge and transfer the clear NaOH layer into microcuvettes and determine the fluorescence, exactly 10 min after the addition of NaOH, in a fluorimeter with excitation and emission, wave lengths set at 396 nm and 522 nm respectively. Since the fluorescence of the metabolites in alkaline solution decreases with time, the fluoresence readings must be taken quickly and at a fixed time.

A standard graph prepared once with authentic 3 – HOBP can be used for calculations if the spectrophotofluorimeter is adjusted with a standard quinine sulfate every time,

b. Fluorimetric method

Principle :

The direct assay is based on the fact that in alkaline solution the substrate (BP) interfers with fluorescence measurement of the product (3-HOBP) at 522 nm only when excited below 425 nm but not at 465 nm. In contrast, the product 3-HOBP has a strong green fluorescence when excited at 465 nm. Using this differential fluorescence characteristics, under instrumental settings of 465 nm excitation and 522 nm emission, small amounts of the product can be measured in the presence of large amounts of the substrate.

Reagent:

Refer Nebert and Gelboin method

Procedure :

The assay conditions are essentially similar to the previous method except that the reaction is carried out in 15 x 120 mm tubes with shaking (160 cycles/min) at 37°C. After 10 min, stop the reaction by the addition of 0.2 ml of 37% neutral formalin and allow the tubes to stand for 5 min. Add 1.0 ml 1.0 N NaOH to each tube with vortex mixing to suspend the microsomes. Measure the fluorescence in a spectrophotofluorimeter with excitation and emission wavelengths of 465 nm and 522 nm respectively. Prepare a standard graph by adding varying amounts of authentic 3-HOBP to 2.2 ml of fixed alkaline microsomal suspensions containing 80 n moles of benzopyrene. Use a working standard of 1 μg/ml of quinine sulfate in 0.1 N H_2SO_4 for calibrating the instrument daily.

References :

1. J. Biol. Chem. **243**, 6242 (1968).
2. Biochem. Biophys. Res. Commun. **70**, 474 (1976).

10. CATALASE

Principle :

The oxygen liberated by the action of the enzyme on hydrogen peroxide is measured manometrically.

Reagents:

1. 0.1 M Phosphate buffer, pH 7.0.

2. Enzyme preparation: 10 mg of fresh tissue is crushed in a power driven tissue grinder using a teflon pestle with 4 ml of 0.1 M phosphate buffer for 2 min at 5°C. The homogenate is then centrifuged for 10 min at 0°C at 1000 rpm in a refrigerated centrifuge and the supernatant is used for the assay.

3. 0.2 M H_2O_2: 1.0 ml of H_2O_2 solution (20 volumes) is diluted to 8.0 ml with distilled water. The solution is freshly prepared.

Procedure:

The conventional Warburg respirometer is used with air as gas phase. In the main compartment of the vessel is added 0.2 ml of 0.2 M H_2O_2 and 2.5 ml of 0.1 M phosphate buffer of pH 7.0. The enzyme preparation is tipped from the side arm after equilibration of the system for 5 min at 24°C. Manometric readings are taken at 1 min intervals for 4 min, which would be linear with time.

Calculation:

Catalase activity is expressed as μl O_2 liberated/mg liver N/hr at 24°C.

References:

1. Methods in Enzymology. Vol II. p. 764 (1956).
2. Ph. D. Thesis (1965) D.S. Sankar Rao, National Institute of Nutrition, ICMR, Hyderabad, India.

11. CYTOCHROME OXIDASE

Principle:

The rate of oxidation of ferrocytochrome c is measured by following the decrease in the absorbancy of its α band at 550 nm in a spectrophotometer.

Reagents:

1. 1 M Potassium phosphate buffer, pH 7.4.

2. Cytochrome c, from horse heart, Type III (Sigma Chemical Co., USA).

3. 1% Reduced cytochrome c solution: 10 mg/ml solution of cytochrome c is prepared in water and reduced with crystalline potassium ascorbate. Four mg of potassium ascorbate are added per mg of cytochrome c. The reduced cytochrome c is dialysed in cold against 10 mM potassium phosphate buffer, pH 7.4, for 18 hr with three intermittent changes of buffer. After dialysis, the solution is used immediately.

4. 0.05 M Potassium ferricyanide solution. Always prepare freshly.

5. 1 mM Potassium cyanide solution: Prepare freshly by neutralising with equimolar potassium dihydrogen phosphate.

6. Mitochondrial enzyme preparations diluted in 10 mM potassium phosphate buffer, pH 7.4 to a protein concentration of 1.0 mg/ml.

Procedure :

The reaction mixture, in a final volume of 3.0 ml has 66 mM potassium phosphate buffer, pH 7.4, 20 μM reduced cytochrome c and 20 μg of mitochondrial protein. The reaction is followed at 30°C for 2 min after the addition of mitochondrial fraction. Readings are recorded at 15 sec interval. Blank cuvette contains 66 mM potassium phosphate buffer. Absorbance of completely oxidised cytochrome c is determined by adding 0.05 ml of 0.05 M potassium ferricyanide after following the reaction for 2 min. Assays are always carried out within 3 hr after the isolation of the mitochondrial fraction.

Cyanide inhibition : Inhibition of cytochrome c oxidase activity is determined by using 6.66 μM concentration of cyanide. The reaction mixture is preincubated with cyanide for 5 min.

Linearity of oxidation of cytochrome c : In all assays, for comparison of specific activity of different mitochondrial preparations, cytochrome c concentration is fixed at 20 μM. Rate of oxidation of cytochrome c at 20 μM is linear with time and follows first order kinetics. Linearity of reaction rate with enzyme concentration and cytochrome c concentration is established in preliminary experiments.

Kinetics of cytochrome oxidase: Activity determinations are made at 5, 10, 20 and 30 μM concentrations of cytochrome c. Km (apparent) and V max are obtained from a Lineweaver - Burk plot of $(\text{ferrocytochrome c})^{-1}$ vs $(\text{K x cytochrome c})^{-1}$.

Calculation :

The cytochrome c oxidase activity is best expressd as the first order velocity constant for the oxidation of cytochrome c For calculation of cytochrome c concentration, a millimolar extinction coefficient of 19.1 is employed for reduced vs oxidised cytochrome c at 550 nm. The velocity constant is calculated using the equation,

$$K = \frac{\log At_2 - \log At_1}{t_2 - t_1} \times 2.3 \text{ sec}^{-1}$$

where, At_1 and At_2 are the absorbance differences between reduced and completely oxidised cytochrome c at time t_1 (15 sec) and t_2 (75 sec). The specific activity in n moles of cytochrome c oxidase/mg

$$\text{protein/min} = \frac{\text{K (concentration of cytochrome c)}}{\text{concentration of protein}}.$$

Note :

The extent of reduction of cytochrome c is tested by measuring the ratio of the absorbance at 550 nm to the absorbance at 565 nm. It has been suggested that when the ratio of A550/A565 is less than 6.0, the proportion of reduced cytochrome c is too small to be used in the cytochrome c oxidase assay. All the cytochrome c solutions prepared should be tested for this ratio before use.

Reference :

Methods of Biochemical Analysis. D. Glick (Ed.), Inter Science Publishers, Inc. New York, Vol. II, p. 427 (1955).

12. CYTOCHROME P-450

Principle:

The reduced form of cytochrome P - 450 (Cyt P-450) readily combines with carbon monoxide (CO)

to form a complex having an absorption maximum at 450 nm when read against its reduced form not treated with CO. The CO difference spectrum of dithionite reduced sample is used for determining Cyt P-450 using the extinction coefficient difference of 91 cm^{-1} mM^{-1}.

Reagents :

1. 0.10 M Potassium phosphate buffer, pH 7.4
2. Sodium dithionite : Good quality dithionite after checking for its ability to reduce must be used.
3. Carbon monoxide: CO can be generated freshly by the action of concentrated H_2SO_4 on formic acid and purified by passing through KOH - dithionite solution.

Procedure:

Treat microsomal suspension in phosphate buffer (1.0 mg protein/ml) with a few grains of sodium dithionite and take it in the reference and sample cuvettes (or in the two halves of the split cuvette) and record the baseline from 400-510 nm. Transfer the contents of the sample cuvette into a tube and bubble gently with CO for 60 sec. Transfer back to the sample cuvette and record the difference spectrum from 400 - 510 nm again. The difference in absorbance between 450 nm and 490 nm can then be used for the calculation of Cyt P-450 content using the extinction coefficient difference. ($\triangle$E 450-490 nm of 91 cm^{-1} mM^{-1}).

Reference:

J. Biol. Chem. **239**, 2370 (1964).

13 ESTERASE

Principle:

The activity of the enzyme esterase which splits simple ester in biological systems is measured by titrating the acid released.

Reagents:

1. 1% Phenolphthalein in alcohol
2. N/20 NaOH
3. M/3 Potassium phosphate buffer, pH 7.0 : Mix 10 ml M/3 K_2HPO_4 and 3 ml M/3 KH_2PO_4.

Procedure:

To 1 ml of serum in a conical flask is added 3 ml distilled water, 1 ml absolute ethyl butyrate and 0.5 ml M/3 potassium phosphate buffer. The contents are mixed by shaking and kept in an incubator at 37°C for 24 hr. At the end of the incubation period, 5 drops of 1% phenolphthalein are added and the solution titrated against N/20 NaOH till a permanent faint pink colour appears. A control tube is similarly carried through but without the addition of serum.

Calculation :

$$\text{Units per ml serum} = \frac{\text{ml N/20 NaOH for test} - \text{ml N/20 NaOH for control}}{2}$$

1 Unit = the acid released to neutralise 1 ml of N/10 NaOH.

Note:

The activity remains practically unaltered for about two weeks, if kept in cold.

Reference:

Am. J. Physiol. **100**, 266 (1932).

14. GAMMA GLUTAMYL TRANSFERASE

Principle:

Gamma glutamyl transferse (γGT) catalyses the transfer of the gamma glutamyl group from gamma glutamyl peptides to suitable acceptors. In the reaction rate analysis method the artificial substrate γ-glutamyl-p-nitroanilide is used.

γ-Glutamyl-p-nitroanilide + gly-gly ⟶ p-Nitroaniline + γ-glu-gly-gly.

The p-nitroaniline released is monitored by following the increase in absorbance at 405 nm.

Reagents:

1. 115 mM Tris-138 mM glycylglycine buffer: Dissolve 6.96 g Tris and 9.11 g glycylglycine in about 400 ml water, adjust the pH to 8.5 at 37°C with 0.5 N NaOH and make upto 500 ml with water. (Stable for 3 months at 4°C).

2 104 mM γ-Glutamyl-p-nitroanilide solution: Dissolve 29.5 mg γ-glutamyl-p-nitroanilide per ml of 0.5 N HCl just prior to assay and use within 24 hr.

Procedure:

Add 1 ml buffer and 50 µl sample into the cuvette, equilibrate to 37°C (in the cuvette chamber). Add 100 µl of γ - glutamyl-p-nitroanilide solution and mix. Follow the reaction rate at 37°C by monitoring continuously the increase in absorbance at 405 nm (for 5-10 min). Employ everyday a substrate blank in which water is substituted for sample to correct for the spontaneous decomposition of the substrate.

Calculation:

γ GT activity (U/l) = $A_{405\ nm}$ / min x 2323.

Reference:

Clin. Chem. **20**, 1121 (1974).

15. GLUTAMIC - OXALOACETIC TRANSAMINASE IN ERYTHROCYTES (EGOT) AND STIMULATION BY PYRIDOXAL - 5' - PHOSPHATE

Principle:

The determination of GOT activity is based on the transamination of aspartic acid to α - ketoglutaric

acid. One of the products of the reactions, namely, oxaloacetate is converted to pyruvate which is measured colorimetrically.

Since pyridoxal-5' - phosphate is the coenzyme for transaminases, the *in vitro* stimulation of the transaminase activity may be used to determine the saturation of pyridoxal-5' - phosphate of erythrocytes which determines the vitamin B_6 nutritional status.

Reagents:

1. 0.89% NaCl solution
2. 1% Heparin in 0.85% NaCl solution
3. 2 mg Pyridoxal-5' - phosphate (PLP) in 4 ml of glass distilled water
4. DL - Aspartic acid - α-ketoglutaric acid reagent: Dissolve the following ; 3.61 g DL - aspartic acid, 2.00 g KH_2PO_4 and 0.60 g α - ketoglutaric acid in glass distilled water. The pH is adjusted to 7.4 with 3 N KOH and volume is made upto 100 ml.
5. 100% TCA
6. Aniline-citrate solution : 2 g citric acid is dissolved in 2 ml distilled water and 2 ml of freshly distilled aniline is added.
7. 2, 4 - Dinitrophenylhydrazine (2, 4 - DNP) : Dissolve 100 mg of 2, 4 - DNP in 20 ml conc. HCl and make the volume to 100 ml with distilled water.
8. 2.5% Alcoholic KOH solution : 2.5 g KOH are dissolved in 100 ml of 95% alcohol. The solution must be completely clear.
9. Standard pyruvate (200 μg/ml) : 25mg of sodium pyruvate which is equal to 20mg of pyruvate, is dissolved in 100 ml of distilled water.

Procedure :

Preparation of erythrocyte haemolysate : Blood is collected in heparinised centrifuge tubes and centrifuged at 2000 rpm for 10 min. Serum is removed and the erythrocytes are washed twice by adding 8 - 10 ml of 0.89% NaCl solution each time, mixed and centrifuged for 15 min at 2000 rpm. Finally, volume is made up to the original volume of blood with 0 89% NaCl solution. Erythrocyte count is determined in a haemocytometer. A nineteen-fold volume of glass distilled water is added to the erythrocyte suspension (0.2 ml erythrocyte suspension + 3.8 ml glass distilled water) and kept for 30 min or 1 hr. If samples are not used immediately, they are stored frozen at - 20°C.

With all samples, duplicate analysis should be carried out. Haemolysates are shaken well; cell membranes are allowed to settle and 0.5 ml is taken in centrifuge tubes according to the following scheme :

Blank (B) in duplicate 0.5 ml haemolysate, sample (S) in duplicate 0.5 ml haemolysate and activated sample (A) in duplicate 0.5 ml haemolysate + 0 1 ml PLP.

The above tubes are incubated for 30 min at 37°C, preferably in a shaker water bath at low speed. All the samples are adjusted to 1 ml by adding 0 5 ml and/or 0.4 ml of glass distilled water. The reaction in the blank tubes is stopped by adding 2 drops each of 100% TCA and aniline citrate solution. 0.5 ml aspartate - α - ketoglutarate reagent is added to all tubes and mixed well. The tubes are incubated at 37°C for 30 min preferably in a shaker water bath at low speed The reaction is stopped by adding 2 drops each of 100% TCA and aniline-citrate solution. The reaction in S and A tubes of the same sample should

be stopped together. All tubes are shaken well and left at room temperature for 20 min. 2, 4 – DNP solution 0.5 ml is added to all tubes and mixed. The tubes are left at room temperature for 5 min. Two ml toluene is added and the tubes shaken vigorously. All tubes are centrifuged for 5 min at 2000 rpm. One ml toluene layer is pipetted out and 3 ml alcoholic KOH is added and mixed thoroughly. Extinction is measured at 510 nm against a blank prepared by adding 3 ml alcoholic KOH to 1 ml toluene.

Standard pyruvate, 0, 5, 10, 20, 40, 60, 80 and 100 μg pyruvate in 1 ml are taken and processed as per procedure described above except the addition of 100% TCA. Standard graph is drawn by plotting concentrations of pyruvate against the corresponding extinction.

Calculations:

The EGOT activity equivalent is the amount of pyruvate which has been formed per unit time. The concentration of pyruvate in samples as measured from the standard graph is calculated as μ moles pyruvate/hr/10^8 cells. Molecular weight of pyruvate is 88.07. The stimulation due to addition of PLP is also calculated and expressed as a percentage increase of activity.

If 0.2 ml erythrocyte suspension is diluted to 4 ml and from this 0.5 ml is taken for assay, then,

$$S \text{ or } A = \frac{\mu g \text{ pyruvate}}{88.07} \times \frac{60}{30} \times \frac{4}{0.5} \times \frac{1}{200} \times \frac{10^8}{N_E}$$; where S is the enzyme activity of the sample, A is the enzyme activity in the presence of PLP and N_E is the No. of erythrocytes/μl.

$$\% \text{ Increase in activity} = \frac{A}{S} \times 100 - 100.$$

References:

1. Am. J. Clin. Pathol. **26**, 1101 (1956).
2. Ibid **27**, 235, 1957.

16. GLUTAMIC - OXALOACETIC TRANSAMINASE AND GLUTAMIC - PYRUVATE TRANSAMINASE IN SERUM

Procedure:

Serum glutamic-oxaloacetic transaminase (SGOT) activity is determined in the same way as the erythrocyte haemolysate GOT, except that in place of 0.5 ml haemolysate, 0.5 ml serum is used.

Serum glutamic-pyruvate transaminase (SGPT) activity is determined in the same way as SGOT except for two points.

(a) In place of aspartate – α – ketoglutarate reagent, alanine – α – ketoglutarate reagent is used which is prepared by mixing 2.4208 g DL – alanine, 2.0 g KH_2PO_4 and 0.6 g α – ketoglutarate.

Dissolved in glass distilled water and the pH is adjusted to 7.4 with 3 N KOH and volume is made upto 100 ml.

(b) Addition of aniline-citrate is omitted.

Calculation:

Units of SGOT and SGPT are expressed as μg pyruvate liberated/ml serum, under the conditions of incubation, ie. at 37°C for 30 min.

17. GLUTATHIONE REDUCTASE

a. Colorimetric method

Principle:

The enzyme glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH).

$$GSSG + NADPH + H^+ \xrightarrow{FAD} 2\,GSH + NADP^+$$

The red blood cell glutathione reductase is NADPH dependent and has FAD as cofactor. The enzyme activity is assayed colorimetrically by measuring reduced glutathione formed.

Reagents:

1. Tris buffer, pH 7.4 — 291 μmoles/ml
2. EDTA — 50 μmoles/ml
3. FAD — 60 μmoles/ml
4. GSSG — 10 μmoles/ml
5. NADPH — 10 μmoles/ml
6. 3% Metaphosphoric acid (MPA) in glass distilled water
7. NaCl saturated solution
8. 2% Sodium nitroprusside in glass distilled water. Store in brown bottle.
9. Na_2CO_3-NaCN solution: Dissolve 15.9 g Na_2CO_3 and 0.328 g NaCN in 100 ml glass distilled water.
10. Standard reduced glutathione 50 μg/ml in 3% MPA saturated with NaCl.

Procedure:

Add 0.7 ml glass distilled water to 0.1 ml of RBC (washed and stored at –20°C). Keep for 1 hr. To three sets of tubes (blank, test, stimulated) add 0.12 ml of tris buffer, pH 7.4, 0.05 ml EDTA solution and 0.1 ml haemolysate. Add 0.05 ml FAD solution to the tubes where stimulated activity is measured. Add glass distilled water to make the volume to 0.45 ml. Incubate the tubes for 30 min at 37°C. Add 2 ml MPA to the blank tubes. Add 0.1 ml of GSSG and 0.1 ml NADPH to all the tubes. Incubate the tubes for exactly 15 min at 37°C. Add 2 ml MPA to blank and test. Add solid NaCl crystals to saturate the solution in all the tubes. Mix well and filter.

To 1 ml of filtrate add 3 ml of saturated NaCl solution and keep for 20 min in a water bath at 20°C. Read the absorbance at 520 nm immediately after the addition of 0.5 ml each of sodium nitroprusside and sodium carbonate-cyanide solution.

Calculation:

$$\mu\text{moles GSSG reduced/ml RBC/hr} = \frac{\text{OD (test-blank) x conc. of standard}}{\text{OD standard}} \times \frac{2.55}{1} \times \frac{80}{2}$$

Note :

Tentative guidelines for interpretation.

Activity coefficient $<$ 1.2 acceptable

between 1.2-1.4 marginally deficient

$>$ 1.4 deficient.

In riboflavin deficiency enzyme activity decreases, but can be stimulated by the *in vitro* addition of FAD. The *in vitro* stimulation with the addition of FAD can be used for assessing riboflavin nutritional status.

b. Spectrophotometric method

Principle :

Spectrophotometric method of glutathione reductase is same as that of colorimetric method, except for the measurement of NADP formed.

Reagents :

1. Phosphate buffer, pH 7.4

(a) Dissolve KH_2PO_4 2.722 g in distilled water and make upto 200 ml. Store at 4°C.

(b) Dissolve $K_2HPO_4.3H_2O$ 22.8 g in distilled water and make upto 1 l. Store at 4°C.

The two components are allowed to reach room temperature. Pour 100 ml of component 'b' into a beaker and titrate with component 'a' to a pH of 7.4. Store buffer in refrigerator at 4°C.

2. 0.1 M Sodium bicarbonate solution : Dissolve 8.4 g $NaHCO_3$ in distilled water and adjust the volume to 1 l. Store at 4°C, prepare fresh every 14 days.

3. 50 mM GSSG solution : Weigh 155 mg of GSSG into a small beaker. Add 5 ml of distilled water and 50 μl of 0.8 M NaOH. Mix well and place in an ice bath. Prepare fresh daily.

4. 250 μ M Flavin adenine dinucleotide (FAD) solution : Weigh 1.0 mg of FAD into a small beaker. Add 5 ml of distilled water, mix well and put immediately in the dark. Prepare fresh daily.

5. 4 mM NADPH solution : Weigh 33.2 mg NADPH (tetrasodium salt) into a small beaker. Add 10 ml of 1% sodium bicarbonate solution, mix well and put into an ice bath. Prepare fresh daily.

6. 80 mM EDTA solution : Dissolve potassium-EDTA (3.2 g) in distilled water and make up to 100 ml. Prepare fresh weekly and store at 4°C.

7. Preparation of haemolysate : To 200 μl of the frozen red cells, add 3.0 ml of phosphate buffer. Mix well and centrifuge.

Procedure :

Tubes are set up as follows :

Reagent	Tube (I) (ml)	Tube (II) (ml)
Phosphate buffer	2.0	2.10
Haemolysate	0.10	0.10
GSSG	0.10	0.10
FAD	0.10	—
EDTA	0.05	0.05

The tubes are incubated at 37°C for 15 min following which 0.1 ml of NADPH solution is added to each tube. The reaction rate is then continuously monitored at 340 nm for 5 min and the linear absorbance change is measured. Read tubes I and II. The whole 5 min period should be monitored, when appropriate a 3 sec dwell time may be used.

△ A over the whole 5 min period will usually be used for calculation purposes if during this time the graph is linear.

Calculations:

One unit of the activity is expressed as the μmoles of NADPH oxidised/hr/ml of red cells.

△ A= decrease in absorbance in one min at 340 nm. Molar extinction of NADPH= 6.22×10^3.

$$\text{EGR activity} = \frac{\triangle A \times 2.45 \times 16 \times 60}{6.22 \times 0.1} = \triangle A \times 3781 \text{ U/ml}$$

Results should be expressed as basal and stimulated activity. The activity coefficient (AC) is the ratio of stimulated to basal activity.

References :

1. J. Clin. Invest. **48**, 1957 (1969).
2. Science **165**, 613 (1969).
3. J. Clin. Invest. **48**, 7a (1969).
4. Clin. Chem. **22**, 327 (1976).
5. J. Nutr. **101**, 1459 (1971).

18. GLUTATHIONE - S - TRANSFERASE

Principle :

Glutathione-S-transferase activity is measured by following the increase in absorbance at 340 nm, using 1-chloro-2, 4-dinitrobenzene as the substrate.

Reagents :

1. 0.3 M Phosphate buffer, pH 6.5
2. 30 mM Glutathione
3. 30 mM 1-Chloro-2, 4-dinitrobenzene (CDNB prepared in 95% alcohol).
4. Enzyme source : Prepare 10% liver homogenate and centrifuge at 105.000 g for 60 min. Use the supernatant after appropriate dilution as the enzyme source (approx. protein concentration, 100 μg/ml).

Procedure :

Take the following solutions in a quartz cuvette in the given proportion :

One ml of phosphate buffer, 0.1 ml CDNB and 0.1 ml enzyme. Adjust the volume to 2.9 ml with glass distilled water. Preincubate the reaction mixture in the thermostated cell compartment of the Gilford spectrophotometer, at 37°C for 5 min. Start the reaction by the addition of 0.1 ml of

30 mM glutathione. Follow the absorbance for 5 min at 340 nm. Reaction mixture without the enzyme is used as the blank.

Calculation:

$$\mu \text{ moles of CDNB-GSH conjugate formed/min/mg protein} = \frac{\text{OD} \times 3 \times 1000}{9.6 \times 5 \times \text{protein in mg}}$$

9.6 is the difference in the millimolar extinction coefficient between CDNB GSH conjugate and CDNB.

Note:

See that the increase in OD does not go beyond 0.05/min. The reaction should be linear at least for 3 min.

Reference:

J. Biol. Chem. **249**, 7130 (1974).

19. LACTIC DEHYDROGENASE

Principle:

The reduction of NAD is followed at 340 nm in a spectrophotometer. It is a direct measure of oxidation of lactate to pyruvate.

Reagents:

1. 0.5 M Sodium lactate
2. 0.02 M NAD
3. 0.1 M Glycine buffer, pH 10.0

Procedure:

The reaction is started by the addition of 0.1 ml of serum to a 3.0 ml quartz cuvette containing 0.5 ml of sodium lactate, 0.1 ml NAD and 2.0 ml of glycine buffer and the increase in extinction at 340 nm is followed at 30 sec intervals, for 2 min. Lactic dehydrogenase activity is expressed as the change in OD/min/ml serum.

Reference:

J. Lab. Clin. Med. **48**, 607 (1956).

20. LIPASE

a. Serum

Principle:

The activity of serum lipase is measured by estimating titrimetrically the acid released.

Reagents:

1. Tributyrin
2. 2% Calcium acetate in distilled water
3. 0.5% Sodium barbiturate in distilled water
4. Gum acacia
5. 1% Phenolphthalein in alcohol
6. N/20 NaOH
7. 0.0037 M Sodium tauroglycocholate

Procedure:

To 10 ml of the freshly prepared tributyrin emulsions (2.9 ml tributyrin + 50 ml 2% calcium acetate + 50 ml 0.5% sodium barbiturate + 200 mg gum acacia) is added 0.5 ml of sodium tauroglycocholate and 0.5 ml serum. The contents are mixed by shaking and kept in an incubator at 37°C for 4 hr, at the end of which 5 drops of 1% phenolphthalein are added and the mixture titrated against N/20 NaOH. A reagent blank is also titrated, but without the addition of serum.

Calculation:

$$\text{Units/100 ml serum} = (\text{ml of N/20 NaOH for test} - \text{ml of N/20 NaOH for control}) \times \frac{100}{0.5} \times \frac{1}{2}$$

1 Unit = the acid released to neutralise 1 ml of N/10 NaOH.

Note:

The activity remains unaltered for about two weeks, if the sample is stored in the cold.

Reference:

J. Lab. Clin. Med. **40**, 860 (1952).

b. Tissues

Principle:

The free fatty acids liberated by the lipolytic activity of the tissue homogenates incubated with triolein or other naturally occurring oils can be estimated by complexing with copper reagent and subsequent colour development.

Reagents:

Reaction mixture or substrate: The reaction mixture has the following composition. 20mg of oil or synthetic glycerides, 5 ml of 1.0 M triethanolamine (pH 8.5), 2.5 ml of 20% BSA and 2.5 ml of 0.1 M NaCl. It is emulsified prior to enzyme reaction in a hand homogeniser.

Tissue preparation: Tissues are homogenised in a medium containing 0.25M sucrose and 10mM tris HCl at pH 7.4. The homogenates are suitably diluted to have 10 mg protein/ml. This preparation serves as the source of lipase.

Procedure:

The lipolytic activity is determined by adding 0.25 ml of diluted homogenate to 0.75 ml of pre-incubated reaction mixture at 37°C for 15 min. The incubation is carried out for about 20 min with

shaking in Dubnoff shaker maintained at 37°C. The reaction is stopped by adding 8 ml of 2 : 1 chloroform-methanol mixture. The activity is determined colorimetrically by the procedure of Duncombe (Refer Lipids under Free fatty acids). Protein is estimated in the diluted homogenate by the method of Lowry (Refer Proteins, amino acids and metabolites).

Calculation :

Specific activity = μ moles of FFA liberated/mg protein/hr.

Reference :

Biochem. J. 88, 7 (1963).

21. NICOTINATE PHOSPHORIBOSYL TRANSFERASE

Principle :

The principle is based on the measurement of radioactive niacin ribonucleotide formed from radioactive niacin and PRPP in the presence of ATP.

Reagents :

1. 0.2 mM Niacin (carboxyl-^{14}C) : 1.78 μCi/μmole
2. 0.005 M $MgCl_2$
3. 1M Tris-acetate buffer, pH 7.5
4. 0.5 mM ATP
5. 0.4 mM NaF
6. 1.0 mM PRPP
7. Bray's mixture : Refer Appendix

Procedure :

Tissue preparation : The procedure for the preparation of liver nicotinate phosphoribosyl transferase is the same as described for the enzyme quinolinate phosphoribosyl transferase.

The incubation mixture for the enzyme assay contains, niacin (carboxyl-^{14}C) 0.1 ml (20 n moles), tris-acetate buffer 0.2 ml (200 n moles), $MgCl_2$ 0.1 ml (100 n moles), NaF 0.1 ml (40 μ moles), PRPP 0.1 ml (100 n moles), ATP 0.1 ml (5 μ moles) and enyzme 0.1 ml. Water is added to make up the volume to one ml. Addition of PRPP is omitted in blank.

The tubes containing the assay mixture are incubated in a metabolic shaker water bath at 37°C for 45 min. The tubes are then removed and kept in a boiling water bath for 1 min to arrest the reaction. After cooling, the tubes are centrifuged to remove the denatured protein. Half ml of the clear supernatant is then passed through a small column (1.0 x 0.5 cm) of Dowex (50 x 4) resin, and eluted with 2.5 ml of water. Under these conditions, radioactive niacin ribonucleotide passes through the column whereas niacin is retained on the column. Half ml of the effluent is counted in Bray's mixture.

Calculation :

The activity of nicotinate phosphoribosyl transferase is expressed as n moles of niacin ribonucleotide formed/hr/mg protein.

References :

1. J. Biol. Chem. **240**, 1395 (1965).
2. Biochem. Biophys. Acta **85**, 255 (1964).
3. Biochem. Med. **12**, 41 (1975).

22. PICOLINATE CARBOXYLASE

Principle :

The substrate α-amino-β-carboxymuconic-ξ-semialdehyde is generated *in situ* from 3-hydroxyanthranilic acid by the addition of the enzyme 3-hydroxyanthranilate oxygenase. The assay of picolinate carboxylase is based on the measurement of decrease in the absorbance of α-amino-β-carboxymuconic-ξ-semialdehyde at 360 nm. The product formed, α-aminomuconic-ξ-semialdehyde, undergoes spontaneous cyclisation to form picolinic acid and this does not affect the overall reaction.

Reagents :

1. 0.7 mM 3-Hydroxyanthranilic acid : 1.3 mg dissolved in 1 ml of 0.6N H_2SO_4 and then diluted to 12.5 ml with water. This is prepared fresh just before use and protected from light.
2. 0.1 M Phosphate buffer pH 7.5.

Procedure :

Procedure for kidney picolinate carboxylase preparation is the same as described for quinolinate phosphoribosyl transferase.

Preparation of acetone powder of 3-hydroxyanthranilate oxygenase: Adult rat livers are homogenised in 0.01 M phosphate buffer (pH 6.5) at 4°C. The homogenate is then slowly added to 10 volumes of dry acetone kept at –15°C with rapid mechanical stirring. This suspension is held at -15°C for about 10 min. The precipitate is collected by rapid filtration on a Buchner funnel which is maintained at –15 °C. The precipitate is washed, thrice, each time using three volumes of cold (-15°C) acetone (calculated from the original homogenate volume). The acetone is removed by using a stream of nitrogen and the powder is dried in a vacuum desiccator over $CaCl_2$.

On the day of use, 0.5 g of acetone powder is treated with 5 ml of 10^{-5} M $FeSO_4$ solution and centrifuged. The supernatant is used as the source of the enzyme, 3-hydroxyanthranilate oxygenase.

Two aliquots (2.8 ml) of the reaction mixture containing 0.5 ml buffer, 0.1 ml acetone powder extract and 2.2 ml water are taken in each of the blank and sample cuvettes of the Beckman DB spectrophotometer. The instrument is adjusted to 100% transmission. The reaction is initiated by the addition of 0.1 ml 3-hydroxyanthranilic acid solution to the sample cuvette. To the blank cuvette 0.1 ml of water is added instead of the substrate. The absorbance at 360 nm is recorded against time. The maximum absorbance at 360 nm reaches within 2 min. At this point 0.1 ml of the enzyme (kidney picolinate carboxylase) is added to both the cuvettes and the rate of disappearance of absorbance at 360 nm is recorded.

Calculation :

Picolinate carboxylase activity is expressed as n moles of substrate disappeared/min/mg protein.

References:

1. J. Biol. Chem. **240**, 1395 (1965).
2. Ph.D. Thesis (1973) Ghafoorunissa, National Institute of Nutrition, ICMR, Hyderabad, India.

23. SODIUM - POTASSIUM ATPase

Principle:

Sodium-potassium adenosine triphosphatase (NaK-ATPase) transports Na^+ and K^+ against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). The liberated Pi is estimated by Fiske and Subba Row method.

Reagents:

1. Enzyme preparation: Isolated membrane preparation (erythrocyte membrane) or microsomal fraction (see Tissue fractionation under Miscellaneous) from tissues are frozen in methanol bath and kept frozen at –20°C, if enzyme activity cannot be assayed immediately. Enzyme preparation is fairly stable for upto 2 weeks.

2. Reaction mixture: Consists of 1 M NaCl 1.40 ml (140 mM), 1 M KCl 0.14 ml (14 mM), 0.1 M Mg Cl_2 0.30 ml (3 mM), 0.2 M EDTA 0.01 ml (0.1 mM), 0.5 M tris HCl pH 7.4 0.40 ml (20 mM) and glass distilled water 2.75 ml (store refrigerated). Use 0.25 ml per assay for a final volume of 0.5 ml or 0.5 ml per assay for a final volume of 1.0 ml.

3. 30 mM ATP: Dissolve 181.8 mg (disodium salt) in 6 ml water. Adjust pH to 7.4 with 0.5 M tris base. Make upto 10 ml with glass distilled water. Keep frozen in small convenient aliquots.

4. Ouabain: 10 mM, 14.57 mg in 2 ml or 2 mM, 14.57 mg in 10 ml water. Store frozen in amber coloured bottles.

5. 10% TCA in water.

6. Reagents for Fiske and Subba Row method for phosphate determination. (Refer Phosphate under Minerals).

Procedure:

Enzyme preparation and ATP solution is thawed and kept on ice. Reaction mixture 0.25 ml is taken in 2 test tubes. 10 mM ouabain 0.1 ml is added to one tube while to the other equal amount of glass distilled water is added. Enzyme preparation 0.05 ml is added to both tubes. (In case of kidney, 75 μg while in case of liver 150 μg of protein). To make total volume of incubation mixture 0.5 ml, 0.05 ml of water is added to both the tubes (after preincubation of 5 min), reaction is initiated with the addition of 0.05 ml of ATP solution. The reaction is terminated after 10 min by adding 0.5 ml of 10% TCA and the tubes are immediately transferred to ice. Enzyme blank is run in a similar way but enzyme protein is added after the addition of TCA. After keeping on ice, for 10 min, the tubes are centrifuged for 5 min to remove the precipitate. Whole supernatant is taken for phosphate estimation by Fiske and Subba Row method.

It is advisable to carry out atleast one standard phosphorus determination with each set of assay. This provides a check on the reagents.

For erythrocyte membrane: About 200 μg of protein is taken for enzyme assay. Ouabain is used at a concentration of 0.2 ml. Incubation period is 60 min for rat and 2 hr for the human preparations. Rest of the procedure is same as described above for other tissues.

Calculation:

The enzyme activity is expressed as μ moles of Pi liberated/hr/mg protein

$$= \frac{\text{Conc. of Pi standard}}{\text{Klett reading for standard}} \times \frac{\text{sample reading}}{\text{mg protein}}.$$

Note:

The difference in the activity in the absence and presence of ouabain is taken as $Na^+ - K^+$ ATPase activity. Activity in presence of ouabain is Mg^{++} ATPase activity.

References:

1. J. Biol. Chem. **66**, 375 (1925).
2. Life Sci. **6**, 595 (1967).

24. SUCCINATE DEHYDROGENASE

Principle:

Succinate dehydrogenase can catalyse the oxidation of succinate by a number of artificial electron acceptors. This property has been made use of in the assay of its activity using phenazine methosulfate (PMS) and 2, 6-dichlorophenol indophenol (DCIP).

The overall reaction is:

$$\text{Succinate} + \text{PMS} \longrightarrow \text{Fumarate} + \text{PMSH}_2$$

$$\text{PMSH}_2 + \text{DCIP} \longrightarrow \text{PMS} + \text{DCIPH}_2$$

Reduction of $DCIPH_2$ can be followed spectrophotometrically at 600 nm

Reagents:

1. 0.2 M Phosphate buffer, pH 7.8
2. 0.6 M Succinic acid readjusted to pH 7.8 with NaOH
3. 0.0015 M DCIP in water
4. 0.009 M PMS in water. Stored in dark and kept frozen. Avoid undue exposure to light.
5. 0.045 M KCN freshly prepared in water
6. Succinate dehydrogenase: Desired preparation like mitochondrial fraction or any other preparation.

Procedure:

Take the following reagents in a 3 ml spectrophotometer glass cuvette. 0.2 M phosphate buffer pH 7.8 0.75 ml (50 mM), 0.045 M KCN 0.10 ml (1.5 mM), 0.6 M succinate 0.2 ml (40 mM), 0.0015 M DCIP 0.1 ml (0.05 mM) and 0.009 M PMS 0 to 0.6 ml (0 to 1.8 mM). Make upto 2.95 ml with water.

The reaction is started by the addition of 0.05 ml of enzyme preparation. The amount of enzyme added should be such that it will produce an absorbance change between 0.05-0.20 per min. The

change in OD (△ 600 nm) against water blank may be recorded after 5 sec. The linearised initial slope is used for calculation of the activity.

Correction needs to be applied for nonenzymatic reaction at each level of PMS without the enzyme preparation.

Calculation :

The observed △ 600 nm is converted to μmoles succinate oxidised = 0.0476 x △ 600 nm.

The specific activity is expressed as μmoles succinate oxidised/min/mg enzyme protein. Vmax values for the artificial electron acceptors are expressed similarly.

Note :

In this assay the activity is fairly linear with enzyme concentration either at fixed concentration of PMS or when extrapolated to Vmax. However, for the reasons which are not clear, these straight lines do not go through the origin even when corrected for nonenzymatic reaction. The main difficulties in this assay are due to deviation from linearity with time and nonenzymatic reaction between PMS and DCIP.

Reference :

Methods in Enzymology, R. W. Egtabrook and M. E. Pullman (Eds). Vol. X, p. 323 (1967).

25. TRANSKETOLASE

a. Colorimetric method

Principle :

The enzyme transketolase (TK) which catalyses some of the reactions of hexosemonophosphate shunt pathway needs thiamine pyrophosphate (TPP) as cofactor.

$$\text{Xylulose-5-phosphate} + \text{Ribose-5-phosphate (R-5-P)} \xrightarrow[\text{TPP}]{\text{TK}}$$

Sedoheptulose-7-phosphate (S-7-P) + Glyceraldehyde-3-phosphate (G-3-P).

In thiamine deficient erythrocytes enzyme activity is low, but can be stimulated by the *in vitro* addition of TPP. The extent of its stimulation with TPP is used for assessing thiamine nutritional status. The enzyme activity can be assayed by measuring the S-7-P formed colorimetrically or G-3-P formed by a coupled enzyme assay.

Reagents :

1. Buffer B : Mix 4 parts saline (0.9%), 103 parts 1.15% KCl, 1 part 3.82% Mg $SO_4.7H_2O$ and 20 parts phosphate buffer, pH 7.4 (17.5 g K_2HPO_4, add 1 N HCl till pH is 7.4. Make up the volume to 1 l).
2. TPP 500 μg/ml : Dissolve in buffer B.
3. 7.5% TCA in glass distilled water.
4. 0.0387 M R-5-P : Dissolve 10.6 mg of R-5-P Na salt in 1 ml of glass distilled water. Stable for 1 week at 4°C.
5. 3% Cysteine hydrochloride in glass distilled water.
6. Standard sedoheptulose anhydride, 105 μg/ml.

Procedure :

Preparation of enzyme: Centrifuge heparinised blood (obtained from fasting subjects) at 3000 rpm for 15 min. Remove plasma and the white cells from the top of the packed cells. Resuspend the cells in ice cold saline and centrifuge at 3000 rpm for 15 - 30min. Remove the supernatant saline and the top layer of cells. Store aliquots of the packed RBC at – 20°C.

Add 0.5 ml of glass distilled water to 0.5 ml RBC and keep for 1 hr. Carry out duplicate analysis with all samples. Take 0.1 ml of haemolysate in three sets of tubes (blank, basal activity and stimulated activity). Add 0.05 ml of TPP solution and 0.1 ml of buffer B to one set of tubes (stimulated activity). Add 0.15 ml of buffer B to the other sets of tubes. Incubate all the tubes at 37°C for 30 min. Add 0.10 ml R-5-P solution to all the tubes. Incubate all the tubes for exactly 30 min. Add 1.5 ml of 7.5% TCA to all the tubes and keep for 15 min. Prepare standard by taking 0.15 ml standard, 0.35 ml glass distilled water and 1.5 ml 7.5% TCA. Centrifuge the tubes at 3000 rpm for 15 min. Take 0.5 ml of supernatant and add 2 ml of ice cold conc. H_2SO_4, shake well and keep in an ice bath for 1 min. Then place the tubes in a boiling water bath for exactly 4 min. Cool the tubes to room temperature and add 0.05 ml of 3% cysteine hydrochloride. Mix well and keep overnight. Measure the absorbance at 510 and 540 nm.

Calculation :

Calculate the difference in OD (510-540) for all the tubes.

$$\text{Enzyme activity, } \mu\text{moles S - 7 - P formed/ml RBC/hr} = \frac{\text{OD test - OD blank}}{\text{OD standard}} \times 3.0.$$

$$\text{Activity coefficient} = \frac{\text{Stimulated activity}}{\text{Basal activity}}.$$

Note :

Tentative guideline for interpretation of activity coefficient: < 1.15 acceptable, 1.15-1.25 marginal deficiency and > 1.25 deficiency.

References :

1. J. Nutr. **71**, 273 (1960).
2. Am. J. Clin. Nutr. **23**, 52 (1970).

b. Spectrophotometric method

Principle :

The erythrocyte transketolase activity (TK) is measured by using ribose-5-phosphate as substrate and the glyceraldehyde-3-phosphate formed is converted to dihydroxy acetone phosphate by triose phosphate isomerase (TIM). The dihydroxy acetone phosphate formed is then reduced to glycerol-1-phosphate by glycerol phosphate dehydrogenase (GDH) and NADH, the latter being simultaneously oxidised to NAD. The rate of decrease in absorbance at 340 nm consequent upon NADH oxidation provides a measure of transketolase activity.

$$\text{Ribose-5-phosphate} + \text{Xylulose-5-phosphate} \xrightleftharpoons{\text{TK}} \text{Sedoheptulose - 7 - phosphate} + \text{Glyceraldehyde - 3 - phosphate.}$$

$$\text{Glyceraldehyde-3-phosphate} \xrightleftharpoons{\text{TIM}} \text{Dihydroxy acetone phosphate.}$$

$$\text{Dihydroxy acetone phosphate} + NADH^+ + H^+ \xrightleftharpoons{\text{GDH}} \text{Glycerol-1-phosphate} + NAD^+.$$

Reagents :

1. 0.1 M Tris-HCl buffer, pH 7.6 : Dissolve 12.11 g of tris in 250 ml of distilled water and add 400 ml of 0.1 M HCl, check the pH and make upto 1 l. Store at 4°C. Stable for one month.

2. 14.4 mM D-Ribose-phosphate solution : Dissolve 0.1 g of D-ribose-5-phosphate disodium salt in 30 ml of tris-HCl buffer solution. Store at 4°C for upto one week.

3. 10 mM Thiamine pyrophosphate solution : Dissolve 0.028 g of thiamine pyrophosphate in 5 ml of tris-HCl buffer. Prepare fresh before use.

4. 5 mM NADH solution : Weigh 0.036 g of NADH into a small beaker. Add 10 ml of tris-HCl buffer solution, mix well and place in an ice bath. Prepare fresh before use.

5. Glycerol-3 - phosphate dehydrogenase - triose phosphate isomerase (GDH-TIM) : Sigma catalogue No. G 6755 sulphate free preparation. To be diluted 1 in 10 with water before use.

Procedure :

Preparation of haemolysate : To 200 μl of the frozen red cells add 1.8 ml of distilled water, immediately mix and centrifuge.

Tubes are prepared as follows :

Reagent		Tube I (ml)	Tube II (ml)	Tube III (ml)
Ribose–5–phosphate buffer	—	2.80	2.80	—
Haemolysate	—	0.10	0.10	0.10
GDH-TIM	—	0.01	0.01	0.01
TPP	—	0.10	—	—
Tris-HCl buffer	—	—	0.10	2.90

The tubes are incubated at 37°C for 15 min following which 0.1 ml of NADH solution is added to each tube, the tubes shaken and placed in the moving cuvette holder. The reaction rate is monitored at 340 nm for 15 min. Tubes I (stimulated) and II (basal) may be read against Tube III (blank) or $\triangle$ A for Tube III is subtracted from that of Tubes I and II. The whole 15 min period should be monitored. The $\triangle$ A after the first 8 min should be used for calculation since the slope is not linear over the first 8 min. A 3 sec dwell time should be used.

Calculation :

One unit of the activity is expressed as the μmoles of NADH oxidised/hr/ml of red cells.
$\triangle$ A=decrease in absorbance in 1 min at 340 nm. Molar extinction of NADH= 6.22×10^3

$$\text{TK activity} = \frac{\triangle A \times 3.11 \times 10 \times 60}{6.22 \times 0.10} = \triangle A \times 3000 \text{ U/ml of RBC.}$$

Results should be expressed as basal and stimulated activity. The activity coefficient is the ratio of stimulated to basal activity.

Reference :

J. Clin. Chim. Acta 33, 379 (1971).

26. TRYPTOPHAN OXYGENASE

Principle:

Tryptophan oxygenase in liver catalyses the conversion of tryptophan to N-formylkynurenine. Liver contains a formylase at a concentration 600 times greater than that of tryptophan oxygenase. Therefore, in the determination of tryptophan oxygenase activity, the kynurenine formed from the combined activities of these two enzymes is measured spectrophotometrically.

Reagents :

1. 0.14 M Potassium chloride in 0.0025 N NaOH.
2. 0.03 M L-Tryptophan : Dissolve 613 mg L-tryptophan in 20 ml of 1%$NaHCO_3$ and pH adjusted to 7.0 and volume made to 100 ml with water.
3. 0.2 M Phosphate buffer pH 7.0.
4. 5% Zinc acetate (W/V) solution (prepared just before use).
5. 0.2N NaOH.

Procedure:

Preparation of the liver homogenate : Liver is homogenised in 9 volumes of ice cold 0.14 M KCl and then centrifuged for 2 min at 600 g to remove the debris. The supernatant is used for the assay of the enzyme.

The incubation mixture containing, phosphate buffer 0.5 ml (100 μ moles), L tryptophan 0.3 ml (9 μmoles), potassium chloride 2.0 ml (280 μmoles) and liver homogenate 2 0 ml is taken in a conical flask and incubated in a metabolic shaker bath at 37°C for 1 hr. The reaction is stopped by the addition of 2 ml of zinc acetate followed by 2.5 ml of 0.2 N NaOH. The blank contains all the reagents except tryptophan, which is added after deproteinisation. The contents of the flasks are mixed well, centrifuged for 5 min and filtered. The OD of the clear filtrate is read against blank at 365 nm.

Calculation :

The concentration (C) of kynurenine is calculated from the OD (E) and extinction coefficient (K) of kynurenine (0.227), ie. $C = E \times K$.

The enzyme activity is expressed as follows,

$$\mu \text{ moles kynurenine formed/hr/100mg N} = C \times \frac{9.3}{2 \times \text{mg N in one ml homogenate}} \times 100, \text{ where,}$$

9.3 is the final volume in ml of the deproteinised incubated mixture and 2 is the volume of homogenate in ml used for the assay.

Note:

Enzyme assay should be carried out within 1 hr after killing the rats. Care should be taken to avoid direct exposure of the reaction mixtures and OD is measured within half an hr after arresting the reaction.

Reference :

Newer Methods of Nutritional Biochemistry, A.A. Albanese (Ed.), Vol. II, p. 270 (1965).

27. QUINOLINATE PHOSPHORIBOSYL TRANSFERASE

Principle :

$$\text{Quinolinic acid} + \text{PRPP} \xrightarrow{Mg^{2+}} \text{NMN} + \text{PPi} + CO_2.$$

The niacin ribonucleotide formed is hydrolysed to niacin with alkali and the niacin released is determined by microbiological assay using *L. arabinosus*.

Reagents :

1. 0.14 M KCl.
2. 4 mM PRPP : 4 mg of magnesium salt of PRPP dissolved in 1 ml of water.
3. 1 mM Quinolinic acid.
4. 0.01 M $MgCl_2$.
5. 0.05 M Tris-acetate buffer, pH 7.0 : Dissolve 6.07 g of tris in 75 ml of water, adjust the pH to 7.0 with glacial acetic acid and the volume made upto 100 ml.

Procedure :

Preparation of enzyme : Liver is homogenised in cold with two volumes of 0.14 M KCl in a glass-teflon homogeniser for 3 min. The homogenate is centrifuged at 20,000 g for 20 min at 2°C. The supernatant solution is treated with charcoal (15 g% W/V) to remove niacin nucleotides. The charcoal is removed by centrifuging at 10,000 g for 10 min and the enzyme assayed in the supernatant.

Incubation mixture consists of quinolinic acid 0.1 ml (100 nmoles), PRPP 0.1 ml (400 nmoles), $MgCl_2$ 0.1 ml (1 μ mole), tris-acetate buffer 0.1 ml (50 μ moles) and water 0.4 ml taken in test tubes and incubated in a water bath at 37°C for about 10-15 min. The reaction is started by the addition of 0.2 ml of the enzyme preparation. Incubation is carried out for 45 min at 37°C in a metabolic shaker water bath. It may be noted that PRPP is not added to the blanks.

At the end of incubation period, the reaction is arrested by heating the tubes for about 30 sec in a boiling water bath. Add 0.2 ml of 0.6 N NaOH and keep in boiling water bath for 30 min.

After alkali hydrolysis, the pH of the incubation mixture is adjusted to 6.8 and the volume is made upto 10 ml. Niacin in the solution is assayed microbiologically (Refer microbiological assay of niacin under Vitamins, coenzymes and metabolites).

The activity of quinolinate phosphoribosyl transferase is expressed as n moles of NMN formed/hr/mg protein.

References :

1. Methods in Enzymology, Colowick, S.P. and Kaplan, O.N. (Eds.) Vol. XVII Part A, p. 491 (1970).
2. Ph. D. Thesis (1973) Ghafoorunissa, National Institute of Nutrition, ICMR, Hyderabad, India.

28. URIDINE DIPHOSPHO GLUCURONYL TRANSFERASE

Principle :

Uridine diphospho glucuronyl transferase (UDPGT) facilitates the transfer of glucuronic acid moiety from uridine diphospho glucuronic acid to an acceptor.

$$\text{p-Nitrophenol} + \text{UDPGA} \xrightarrow{\text{UDPGT}} \text{p-Nitrophenyl-}\beta\text{-glucuronide} + \text{UDP.}$$

UDPGT activity is assessed by following the decrease in p-nitrophenol absorbance at 405 nm as the glucuronide conjugate does not have any absorption at this wavelength.

Reagents :

1. 0.4 M Phosphate buffer, pH 7.4
2. 40 mM $MgCl_2$
3. 1.6 mM p-Nitrophenol
4. 20 mM Uridine diphospho glucuronic acid (UDPGA)
5. 0.2 N TCA
6. 0.5 N NaOH

Procedure:

The assay medium in a volume of 0.4 ml should contain 0.1 M phosphate buffer, 5.0 mM $MgCl_2$, 0.2 mM p-nitrophenol, 0.5 to 1.0 mg of microsomal protein or any other enzyme source and 2.5 mM of UDPGA. Water is substituted for UDPGA in the blank. The tubes are incubated with shaking at 37°C in a water bath for 10 min. The reaction is terminated by the addition of an equal volume of 0.2 N TCA. The tubes are centrifuged at 2500 rpm for 20 min and 0.5 ml aliquot of the supernatant is added to 1.5 ml of 0.5 N NaOH and the absorbance read at 405 nm.

Reference :

Biochem. Pharmacol. **20**, 2703 (1971).

29. XANTHINE OXIDASE

Principle :

Xanthine oxidase activity of the liver is determined by measuring the oxygen consumed using Warburg manometer.

Reagents :

1. 0.05 M Xanthine in 0.05 M NaOH
2. 0.1 M Na-K phosphate buffer, pH 7.4
3. 10% KOH
4. 10% Liver homogenate

Procedure :

One ml of 10% liver homogenate prepared in phosphate buffer, pH 7.4 is taken in each flask. Substrate solution 0.15 ml is placed in the side arm of the flask and 0.2 ml of 10% KOH in the

centre well. Blanks are run side by side without the addition of the substrate (phosphate buffer). Total volume in the main chamber is adjusted to 3.0 ml with the addition of the substrate. Oxygen uptake is measured at 37°C for 100 min using air as gas phase and after an equilibration period of 40 min. The linear rate of oxygen consumption in the presence of substrate is taken as a measure of xanthine oxidase activity which is expressed as μl of oxygen uptake per mg N/hr.

Reference:

Ind. J. Med. Res. **53**, 1087 (1965).

X. DRUGS

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1. ANTIPYRINE

a. GLC method

Principle :

Antipyrine from plasma or saliva is extracted into chloroform after addition of internal standard and alkalinisation and estimated by GLC using flame ionization detector.

Reagents :

1. Antipyrine, stock standard solution : 1.0 mg/ml prepared in glass distilled water.
2. Working standard : 100 μg/ml prepared in pooled serum or water.
3. Phenacetin internal standard : 100 μg/ml prepared in ethylene dichloride.
4. 5N NaOH.

Procedure :

To 0.5 ml sample or standard, add 50 μl (5 μg) phenacetin (internal standard) solution. Add 0.2 ml of 5 N NaOH and mix. Extract by vortex mixing antipyrine and phenacetin internal standard into 2.0 ml of distilled chloroform. Transfer the chloroform layer to fresh tubes and evaporate to dryness under nitrogen. Redissolve in a small volume (20 μ l) of carbondisulfide and inject 0.5 μ l into GLC. Phenacetin and antipyrine elute at 0.5 and 1.0 min respectively under the following conditions.

Equipment : Varian GC 3700.

Column : 50 cm x 1/8" outer diameter stainless steel column packed with 5% OV 101 on Gaschrome Q 80/100.

Temperature : 200°C isothermal. Injection port and detector at 280°C.

Flow rate (ml/min) : N_2 - 30, Air - 300 and H_2 - 30.

Electrometer amplifier at 4 x 10^{-10} amp/mV.

Chart speed : 1 cm/min.

Reference:

J. Pharm. Pharmacol. **25**, 205 (1973).

b. Spectrophotometric method

Principle:

Antipyrine in deproteinised plasma is treated with nitrous acid and the 4-nitroso antipyrine estimated spectrophotometrically at 350 nm.

Reagents:

1. Zinc reagent: Dissolve 100 g $ZnSO_4.7H_2O$ and 40 ml of 6 N H_2SO_4 in water and dilute to 1 l.
2. 0.75N NaOH.
3. 4.0 N H_2SO_4.
4. 0.2% Sodium nitrite.
5. Antipyrine standard: Dilute stock antipyrine standard solution (1mg/ml) to give 2-20 μg/ml concentration.

Procedure:

Take 1.0 ml plasma in duplicate and add 1 ml of water and 1 ml of the zinc reagent. Mix and add dropwise 1.0 ml of 0.75 N NaOH, with continuous vortexing. Vortex for an additional 15 sec and let stand for 10 min. Centrifuge and transfer 1.5 ml of the clear supernatant to clean tubes. Add 50 μl of 4 N H_2SO_4 to all the tubes. Read the absorbance at 350 nm against a water blank containing 50 μl 4N H_2SO_4. Add 50 μ l of 0.2% sodium nitrite solution to each tube noting the time. Read again the absorbance of the solutions at 350 nm exactly 20 min after $NaNO_2$ addition. The 20 min reading after subtraction of the initial reading can then be used for calculating the antipyrine concentration. Standards taken through the whole procedure must be run everyday.

Reference:

J. Biol. Chem. **179**, 25 (1949).

2. CHLORAMPHENICOL AND RELATED NITRO COMPOUNDS

Principle:

Chloramphenicol is an aromatic nitro compound and the method described here involves the quantitative reduction of the aromatic nitro group to form a primary amine which is then determined by diazotisation and subsequent coupling with Bratton - Marshall reagent.

Reagents:

1. 25% TCA.
2. 70% HCl.

3. Stannous chloride : 0.80 g per 100 ml of 70% HCl (prepare fresh).
4. 1% Sodium nitrite : Aqueous solution.
5. 0.5% Ammonium sulphamate : Aqueous solution.
6. Bratton-Marshall coupling reagent : 0.2% aqueous solution of N-(1-naphthyl) ethylene diamine dihydrochloride. (Store in brown bottle in refrigerator).
7. Chloramphenicol standard : Stock solution 100 mg/100 ml.

Procedure :

Place 1 ml of serum or plasma in a centrifuge tube and make up the volume to 4 ml with glass distilled water. Add 1 ml of 25% TCA solution to deproteinise the sample. Allow to stand for a few min and centrifuge at about 2500 rpm for 30 min.

Take around 1.5 ml of the supernatant in each of the two tubes labelled B (blank) to account for the aryl amines, and T (test). To tube B add 0.5 ml of 70% HCl and to tube T add 0.5 ml of 0.8% stannous chloride solution. Place the tubes in a boiling water bath for exactly 20 min. Remove and cool to room temperature.

Add 0.5 ml of sodium nitrite solution to each tube and wait for 2 min, add 2 ml of ammonium sulphamate reagent, mix well by vortex mixer and wait for 5 min. Finally the colour is developed by the addition of 0.5 ml of the coupling reagent. The tubes are maintained at room temperature for 1 hr after which the colour is read at 555 nm against a reagent blank. The standard curve is constructed in the range of 0–30 μg chloramphenicol. The standards are subjected to the same procedure as of the sample.

Calculation :

The difference between the test and blank gives the actual value for the total nitro groups present in the aliquot taken and is calculated for 1 ml of serum.

Note :

The chemical method is not specific for the active antibiotic, since, inactive degradation products of chloramphenicol which still retain the nitro group are also included in the estimation. Hence to increase the specificity of the method for chloramphenicol, the active compound is extracted into an organic solvent.

Two ml of the sample (deproteinised plasma or diluted urine) is pipetted into a 50 ml glass stoppered tube, containing 6 ml of ethyl acetate and 2 ml of 0.2M phosphate buffer at pH 6.0. The tubes are shaken in a mechanical shaker for 15 min. The two phases are allowed to separate and settle down and later the aqueous layer is removed by aspiration and discarded. The organic layer is washed twice by shaking with equal volumes of pH 6.0 phosphate buffer previousiy saturated with ethyl acetate. After separation of the two phases, the ethyl acetate layer is pipetted out equally into two test tubes and evaporated to dryness over a water bath or under nitrogen. The residue is dissolved in 1.5 ml of water and analysed as before, one of the two tubes being used as a blank accounting for the aryl amines.

References :

1. Arch. Biochem. Biophys. **23**, 411 (1949).
2. J. Lab. Clin. Med. **35**, 129 (1950).

3. CHLOROQUINE

Principle :

Chloroquine fluoresces maximally at pH 9.7 with an excitation wavelength of 335 nm and an emission wavelength of 390 nm.

Reagents :

1. 10% NaCl in I N NaOH.
2. 0.1 N HCl.
3. Borate buffer, pH 8.0 : 0.6 M Boric acid in 0.6 M KCl. Adjust the pH to 8.0 by adding 0.6 N NaOH.
4. Borate-alkali : Dissolve 3.09 g H_3BO_3 and 3.73 g KCl in 60 ml IN NaOH. Total volume made upto 100 ml.

Procedure :

Take equal volumes of sample (plasma or whole blood) and 10% NaCl in NaOH and extract chloroquine into 5 ml of ethylene dichloride by mechanically shaking for 30 min. After centrifugation aspirate off the aqueous phase. Wash the solvent phase twice with 5 ml of borate buffer pH 8.0. Each time aspirate off the aqueous phase. Transfer 4 ml of the solvent phase to a test tube. To this add 0.5 ml of 0.1 N HCl and shake for 2 min. Adjust the final pH to 9.7 by adding 0.2 ml of borate-alkali to 0.3 ml of the acid phase. Read the fluorescence in the Farrand fluorimeter using an excitation wavelength of 335 nm and an emission wavelength of 390 nm.

Calculation :

Evaluate the results against chloroquine standards similarly processed and read. The fluorescence of a reagent blank, which is carried through all the operations, is subtracted from that of each unknown.

Note :

The sensitivity of this method is 0.1 μg/ml. The recovery of chloroquine by this procedure is 95 $\pm$ 5 per cent.

References :

1. Antibiot. Chemotherap. **12**, 583 (1962).
2. J. Pharamacol. Exp. Ther. **151**, 482 (1966).
3. Ibid **158**, 323 (1967).

4. PHENYL BUTAZONE

Principle :

Phenyl butazone is extracted into n-heptane from acidified plasma and then into 2.5 N NaOH and estimated spectrophotometrically at 265 nm.

Reagents :

1. 3 N HCl
2. 2.5 N NaOH

3. Standard phenyl butazone in plasma (100 μg/ml) : Prepared in, pooled plasma by incubating with phenyl butazone overnight.

Procedure:

Acidify 1.0 ml plasma or standard in plasma with 0.5 ml 3 N HCl. Extract the drug into 20 ml n-heptane by shaking for 30 min in a metabolic shaker. Transfer 15.0 ml of the heptane layer to a flask and back extract the drug into 5.0 ml of 2.5 N NaOH, by shaking for 20 min. Transfer 4.0 ml of the NaOH layer to silica cuvettes and measure the absorbance at 265 nm against 4 ml of 2.5 N NaOH as blank. Calculate phenyl butazone concentrations by comparing with standards.

Reference :

J. Pharmacol. Exp. Ther. **109,** 348 (1953).

5. SALICYLIC ACID

Principle :

Salicylic acid extracted into chloroform or dichloroethane when treated with ferric nitrate gives a characteristic pink colour of iron-phenol complex and is measured at 540 nm.

Reagents :

1. Protein precipitant : 6 N HCl.
2. 1.67% Ferric nitrate (stock) in 0.07 N HNO_3.
3. 0.084% Ferric nitrate (working solution) : Dilute 25 ml of the stock ferric nitrate solution to 50 ml. This solution should be made fresh.
4. Salicylic acid standard : Dissolve 50 mg of salicylic acid powder in 100 ml of distilled water. To obtain various concentrations of salicylic acid ranging from 10-50 mg% dilute the stock solution appropriately. Use 1 ml of these working standards for estimation. For reagent blank use 1 ml of water.

Procedure :

Pipette 1 ml of standard and/or plasma in 40 ml glass stoppered tubes in duplicates. To each tube add 0.5 ml of 6 N HCl and 10 ml of chloroform or dichloroetahne. Mix each tube for 1 min on a vortex mixer.

Transfer the contents of each tube into a centrifuge tube and centrifuge for 10 min at 1500 rpm. A disc of precipitated protein appears at the interface. Discard the supernatant solution and take the subnatant solution. This can be done by tilting the tube to an angle which makes the disc give way to the subnatant chlorofom or dichloroethane. Pipette 7.5 ml of chloroform or dichloroethane from each of these tubes into another set of stoppered tubes to which 2.5 ml of ferric nitrate working solution is already added. Again mix each tube for 1 min on a vortex mixer. Measure the OD of the supernatant pink solution on a Beckman DU spectrophotometer at 540 nm using the reagent blank to adjust to zero.

Plot the OD of the standards graphically and read off the sample values from the graph.

Reference :

Standard Methods of Clinical Chemistry, D. Seligson (Ed.), Academic Press Inc Publishers. New York and London, Vol III, p. 194 (1961).

6. SULPHONAMIDES

Principle :

Sulphonamides react with nitrite in acid medium to form the corresponding diazonium salts. The excess nitrite is destroyed by ammonium sulphamate and the diazonium salt couples with N- (1-naphthyl) ethylene diamine to form a stable dye.

Reagents :

1. 10% TCA.
2. 0.1% Sodium nitrite (Stable for one week, if frozen).
3. 0.1% Coupling reagent : 3.86 mM N- (1-naphthyl) ethylene diamine dihydrochloride (Stable for one month, if frozen, in a dark coloured bottle. Discard when it turns brown).
4. 0.5% Ammonium sulphamate (prepare fresh every 2 or 3 days).
5. 4 N HCl.
6. Standard : 10 mg of the sulphonamide is dissolved in 100 ml of distilled water (this is stable indefinitely).

Working standard : Pipette 2 ml of stock standard and make up to 50 ml with 10% TCA. This gives a concentration of 4 μg/ml. Dilute appropriately to get concentrations 2, 4, 6, 8, 12 and 16 μg/ml. Make up the volume to 4 ml with distilled water in each tube.

Procedure :

Plasma free sulphonamide : Pipette 1 ml of plasma in a test tube and add 4 ml of 10% TCA and centrifuge. Pipette 0.5 ml of the supernatant into a tube and make up the volume to 4 ml with distilled water.

Plasma total sulphonamide (free and acetyl forms) : Pipette 1 ml of the supernatant obtained as described above and add 0.5 ml of 4 N HCl. To this add 0.5 ml of distilled water. Cover the tube with a marble and place it in a water bath at 100°C for 1 hr. Cool and make up the volume to 2 ml in a graduated tube. Use 1 ml for estimation in duplicates. Then make up the volume in each tube to 4 ml.

Urine sulphonamide : Place 0.5 ml 4 N HCl in a test tube and add 1 ml urine to the tube. Then add 8.5 ml distilled water. Dispense the 10 ml obtained equally in tubes labelled as A and B. Tube A is used for the estimation of free sulphonamide. Tube B is used for the estimation of the total sulphonamide.

Urinary free sulphonamide : Take 1 ml from tube A and make up the volume to 10 ml with distilled water so that the final dilution is 1 : 100. Then take 1 ml for estimation in duplicates and make up the volume in each tube to 4 ml.

Urinary total sulphonamide : Cover the tube B with a marble and place it in a water bath at 100°C for 1 hr. Cool and pipette 1 ml and make up the volume to 10 ml. From this take 1 ml in duplicates for the estimation and make up the volume in each tube to 4 ml. For the reagent blank use 4 ml distilled water. Add 0.8 ml sodium nitrite to all the tubes and wait for 3 min. Then add 0.8 ml of ammonium sulphamate. Shake immediately and wait for 1 min. Finally add 2 ml of coupling reagent. Shake well and read the tubes at 540 nm. Plot the OD of standards graphically and read off the sample values from the graph.

Note :

Sulphonamides exist in blood and urine in two forms, viz, free sulphonamide and acetyl sulphona-

ide. The difference between the concentrations-free and total sulphonamides gives the concentration of cetyl compound.

The colour development reaction is sensitive to light, especially, sunlight. Cover the test tube rack with black paper and also switch off the lights.

Reference :

Clinical Chemistry, Theory and Practice. R. Richterich (Ed.), p. 287 (1969).

. TETRACYCLINE AND DOXYCYCLINE

Principle :

Tetracycline chelates with divalent metal ions such as calcium and an intensely flourescent complex s formed. It is extracted into ethyl acetate in presence of barbital and is fluorimetrically measured.

Reagents :

1. Protein precipitant : 1.8 M TCA and 0.16 M $CaCl_2$.
2. 0.9 M Sodium barbitone solution. (Stable for 2–4 weeks at room temperature).
3. Stock standard solution, 100 μg/ml : Dissolve 10.8 mg of tetracycline HCl or 10.82 mg of oxycycline HCl in 100 ml water.

Procedure :

A standard curve is set up for each batch of determinations. Dilute stock standard solution 50 old to give working standard solution equivalent to 0.5, 1.0, 2, 4, 8 and 10 μg/ml of tetracycline or loxycycline in duplicate tubes. Make up the final volume to 5 ml with water. For the reagent blank ake 5 ml of water. Add 1 ml of protein precipitant and mix well. Take off 2 ml of the solution and dd 4 ml ethyl acetate and 3 ml of sodium barbitone solution. Mix each tube for 1 min on a vortex nixer and allow the phases to separate. Measure the fluorescence of the upper layer on a Farrand fluori-neter using 400 nm as excitation and 520 nm as emission wavelengths.

Pipette 0.5 ml of plasma or 0.1 ml of urine and make up the volume to 5 ml with water. Add ml of protein precipitant and mix well. Centrifuge for about 15 min and take 4 ml of clear supernatant into a tube. Add 4 ml of ethyl acetate and 3 ml of sodium barbitone and proceed in the same way as standards.

Calculation :

Plot the fluorescence of the standards graphically and calculate the concentration of the tests from he graph. Multiply the result obtained by the appropriate factor depending upon the volume of the sample used.

Reference :

Anal. Chem. 33, 862 (1961).

XI. FOOD TOXINS

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1. TRYPSIN INHIBITORS

Principle :

The activity of the enzyme trypsin is assayed using casein as substrate. Inhibition of this activity is measured in the extracts.

Reagents :

1. 0.1 M Sodium phosphate buffer, pH 7.5
2. 2% Casein solution in phosphate buffer
3. Trypsin (E. Merck) solution, 5 mg/ml
4. 0.001 M HCl
5. 5% TCA

Procedure :

Extraction of samples : Four g of the pulverised defatted plant material is treated with 40 ml of 0.05 M sodium phosphate buffer, pH 7.5 and 40 ml of distilled water. The samples are shaken for 3 hr and then centrifuged at 700 g for 30 min at 15°C. The supernatants are diluted in such a way that there is an inhibition between 40 and 60% of the control enzyme activity.

The incubation mixture consists of 0.5 ml of trypsin solution, 2 ml of 2% casein, 1.0 ml of sodium phosphate buffer (pH 7.5, 0.1 M), 0.4 ml HCl (0.001 M) and the extracts 0.1 ml. In all the cases the total volume of the incubation mixture is kept as 4 ml. Incubations are carried out at 37°C for 20 min after which 6.0 ml of 5% TCA solution is added to stop the reaction and corresponding blanks are run concurrently. In this method, one trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance

unit at 280 nm in 20 min for 10 ml reaction mixture under the conditions described and the trypsin inhibitory activity as number of trypsin units inhibited (TUI).

Note :

In order to express the values as specific activities, the total TUI has to be expressed per mg protein. The protein content is generally estimated by Lowry's method. The values for trypsin inhibitory activity must have about 40–60% inhibition in order to have a reproducible and accurate result.

Reference :

J. Agri. Food Chem. **19**, 257 (1971).

2. β – N – OXALYLAMINO – L – ALANINE

Principle :

β-N-Oxalylamino-L-alanine (BOAA) is a neurotoxic amino acid found in *Lathyrus sativus*. It reacts with ninhydrin giving a violet colour, the intensity of which is read in a colorimeter.

Reagents :

1. 70% Aqueous ethanol
2. Buffer system, pH 3.6 : Acetic acid : Pyridine : Water, 5.0 : 0.5 : 94.5
3. 0.1% Ninhydrin in acetone
4. Copper sulphate-ethyl alcohol solution : 300 mg of $CuSO_4$. $5H_2O$ and 50 ml distilled water are mixed with distilled ethyl alcohol just before elution from paper.
5. Pure BOAA solution as standard

Procedure :

Extraction of seeds for BOAA estimation : Two g of powdered *Lathyrus sativus* seeds are treated with 40 ml of 70% aqueous ethanol, stirred at intervals and kept overnight. The contents are centrifuged and the residue is treated in the same way once again. The supernatants are pooled and evaporated to dryness on a boiling water bath at 100°C. The residue is dissolved in 10 ml of distilled water.

Analysis of BOAA : Fifty μl portions of the extract are applied on Whatman No. 1 filter paper strips. Separation is carried out using a Beckman paper electrophoresis cell (Durrum type, Model R) with a buffer system (pH 3.6) and a voltage of 250 is applied for about $4\frac{1}{2}$ hr.

After the run, the strips are dried in an air oven at 80°C. The dry paper strips are sprayed with 0.1% ninhydrin in acetone. They are air dried and the ninhydrin spraying repeated. After air drying, the strips are heated at 80°C for 10 min. Standard BOAA solutions are spotted concurrently on separate paper strips and run along with the samples. Matching with the position of the standard BOAA spot, the corresponding test BOAA spots are eluted individually with 9 ml of mixture of copper sulphate solution and ethyl alcohol. The extracted solution is read in Klett-Summerson colorimeter using green filter (No. 54) and calculations are made after comparison with standard BOAA curves.

Note :

The mobility of BOAA under the specified conditions mentioned above is about 6.7 cm towards

anode and the next moving ninhydrin spot mobility is only 3.1 cm. By this method BOAA at a level of 0.0075 μ moles or 1.32 μg could be estimated. The percent recovery of BOAA is found to be about 95% by this method.

Reference :

Environ. Physiol. Biochem. 5, 172 (1975)

3. AFLATOXINS

Principle:

Methods for the estimation of the aflatoxins in agricultural commodities are based on characteristic fluorescence of these compounds in UV light.

a. Processing of sample

The sample is mixed homogenously and powdered in a Wiley ball mill or any other appropriate mill. Fifty g ground material (or appropriate quantity) is taken into 500 ml conical flask and 25 ml water, 25 g diatomaceous earth (celite) and 250 ml chloroform are added to it. The flask is tightly closed with rubber cork covered with aluminium foil to prevent contact of rubber with chloroform. The contents of the flask are shaken on a wrist-action shaker for 30 min to extract the toxin and filtered through Whatman No. 41 filter paper. The contents are transferred to a bunchner funnel precoated with about 5 mm layer of diatomaceous earth and filtered using light vacuum.

Removal of interfering substances: The chromarographic column consist of a glass tube 22 x 300 mm with a glass stopper at the bottom.

A small plug of glass wool is loosely packed at the bottom of the column and is covered with 5 g anhydrous sodium sulphate. Chloroform is added until the column is about half full. Ten g of silica gel (0.05 – 0.2 mm) is made into a slurry with chloroform and poured in the column. When silica gel has settled, 15 g anhydrous sodium sulphate made as slurry in chloroform is added to the column. As soon as the chloroform is drained off to the top of the sodium sulphate layer, 50 ml of the sample extract to be chromatographed is added to the column. The chloroform is drained off and the column is washed, at a maximum rate, first with 150 ml hexane followed by 150 ml anhydrous diethyl ether. Finally, the aflatoxins are eluted from the column with 150 ml chloroform-methanol (97 : 3 V/V) mixture. The fraction is collected from the time the chloroform-methanol is added until the flow stops. The eluate is evaporated by warming on water bath under a stream of nitrogen. It is desirable to carry out the evaporation in a hood. The residue dissolved in a known volume of chloroform (0.2 or 1.0 ml) is ready for TLC.

b. TLC Method

The standard solutions containing known quantities of aflatoxin B_1 are spotted along with the sample extracts on a TLC plate. The plates are developed ascendingly by dipping them in a chromatographic tank containing chloroform-acetone solvent mixture (95 : 5) to a depth of not more than one cm. It is necessary to saturate the tank with the solvent before use. The solvent front is allowed to reach 10 to 12 cm height. This would take about 30 min at 27°C. After development, the plates are dried in horizontal position and viewed under long wave UV lamp in a dark room or in a cabinet.

Densitometric quantitation:

Spot sample aliquots on TLC plate. Also spot 10 ng of aflatoxin B_1 standard. Develop in chromatographic chamber containing 100 ml of chloroform- acetone (95 : 5). Mark the position of the spots on TLC plate and scan the plate with densitometer. After exciting with 365 nm source, emission is measured at 420 460 nm. The area of the peaks are calculated.

Calculations:

Aflatoxin μg/Kg = (B x Y x S x V)/(Z x X x W), where,

B = Average area of aflatoxin B_1 sample in aliquots
Y = Concentration of aflatoxin B_1 standard μg/ml
S = μl aflatoxin B_1 standard spotted
V = Final dilution of sample extracted (μl)
Z = Average area aflatoxin B_1 peaks in standard aliquots
X = μl sample extract spotted
W = g sample represented in final extract

c. Qualitative method

This technique consists of comparing the intensity of the fluorescent spots of the sample with the corresponding spots of the aflatoxin pure standard. A visual aflatoxin standard of 0.5 μg/ml concentration is used.

Extracted sample is diluted to 0.5 ml or suitable volume in benzene - acetonitrile (98 : 2, V/V) or chloroform. In subdued light, quickly spot on TLC plate 3 levels (5, 7, 10 μl) of sample extract and 3 levels of aflatoxin standard to cover a range of 2-15 ng/spot. Develop the plate to a height of 12-14 cm in suitable solvent. After development the plate is dried in the dark. Under long wave UV compare the fluorescence intensities of the sample spot with those of the standard spots and determine which of the sample spots matches one of the standards. If the sample spot intensity is found to be between those of 2 standard spots the actual intensity should be estimated. If spots of the smallest quantity of sample are too intense to match standards, the sample should be diluted and rechromatographed. Estimates should never be made by extrapolation from the series of standard spots.

Calculations:

Calculate concentration of aflatoxin B_1 in μg/Kg $= \dfrac{S \times Y \times V}{X \times W}$, where,

S = μl aflatoxin standard which matches the unknown
Y = Concentration of aflatoxin B_1 standard μg/ml
V = μl of final dilution of sample extract
X = μl of sample extract spotted giving fluorescent intensity equivalent to S (B_1 standard)
W = g of sample contained in final extract

d. Spectrophotometric method

The presence or absence of blue fluorescent spot corresponding to aflatoxin B_1 at Rf 0.50 to 0.55 and greenish blue fluorescent spot corresponding to aflatoxin G at Rf 0.45 is observed in UV light. The Rf values are checked with the standard aflatoxin run along with the sample extracts.

The area covering the spots or bands are marked by a sharp needle under the UV light and the silica gel covering each spot is scraped carefully with a blade, and collected individually in clean, dry test tubes.

The aflatoxins are extracted with 2 ml cold methanol for 3 min and filtered through glass sinter. It is washed three times with methanol and the combined filtrate is made up to 5 ml. The aflatoxin content is determined by measuring the absorbance at 360 nm in quartz cell. For routine estimations, a standard curve for aflatoxin B_1 from 5 to 50 μg is used. The amount of aflatoxin present in the sample extract is computed from the standard curve.

Calculations :

The aflatoxin content (μg/Kg or ppb) is calculated using the formula,

$$\frac{S \times V \times 5}{A \times W} \times 1000, \text{ where,}$$

W = Weight in g of original sample
V = μl of sample extract prepared for TLC
A = μl of sample extract spotted
S = μl of aflatoxin calculated from the standard curve

e. Chemical confirmation

Aflatoxin B_1, G_1 and M_1 react with trifluoroacetic acid (TFA) to form water adducts and the changed chromatographic properties of their derivatives is the method of confirmation (aflatoxin B_2 and G_2 do not react with TFA).

Spot aflatoxin standard and sample (containing 5-10 ng) on either half of the TLC plate. Cover one half of the plate and superimpose 2 μl TFA on each spot of aflatoxin standard and sample on the other half of the plate. After allowing to react for 5 min, the excess TFA is removed with a hot air blower or placing the plate in an oven at 40°C for 10 min. Develop the plate in chloroform-acetone (9 : 1) with a beaker of water in the TLC chamber.

After development, the plate is air-dried in the dark. Observe under long wave UV light for the altered Rf values of treated aflatoxin and compare with the aflatoxin standards for identification (Rf values of treated aflatoxin B_1 and G_1 are 0.18 – 0.3 and 0.15 – 0.25 respectively).

Note :

Aflatoxin B_1 is a potent carcinogen. Dry, crystalline toxin must be handled in hood using face masks and gloves because it is electrostatic in nature. Aflatoxin solutions must be destroyed by sodium hypochlorite before discarding. Spilled toxin can be destroyed and cleaned by application of sodium hypochlorite. Working area may be covered with disposable material and changed daily.

References :

1. JAOAC. 58, 163 (1975).
2. Tropical products Inst. Rept. G. 70 (1972).
3. Official Methods of Analysis of AOAC; 13th Edn. (1980).
4. Environ. Physiol. Biochem. 3, 13 (1973).

4. T_2 TOXIN

Principle :

T_2 toxins are extracted and purified. They are separated and detected either by TLC or GLC. Since they exhibit no fluorescence nor absorb appreciably in the UV range, they are detected by spraying with

concentrated H_2SO_4. The trimethylsilyl ether of T_2 can be chromatographed in a column of GLC. Derivatisation of the trichothecenes containing hydroxyl groups is important in the analysis of trichothecene by GLC.

a. Processing of sample

Reagents:

1. 30% Ammonium sulfate: Dissolve 300 g $(NH_4)_2SO_4$ in 1 l water.
2. KOH solution, 0.02 N KOH with 1% KCl: Dissolve 1.12 g KOH pellets and 10 g KCl in 1 l water
3. 5% Sodium bicarbonate: Add 5 g $NaHCO_3$ to 100 ml water.

Procedure:

Sample 500 g is powdered and 50 g of the sample is taken for analysis. Add 250 ml methanol-water (1 : 1) and keep for 30 min. Filter through Whatman No. 4 paper. Collect 60 ml of extract and transfer to 500 ml beaker. Add 240 ml of 30% aqueous ammonium sulfate. Stir vigorously for about 1 min, add 100 ml diatomaceous earth and stir for 1 min. Filter through Whatman No. 4 filter paper. Collect 200 ml in graduated cylinder. Transfer it to a 250 ml separating funnel, add 10 ml chloroform and shake vigorously for 1 min. Allow the layers to separate (2.5 min) and collect bottom layer (even if emulsion) in 125 ml separator. Repeat with a second 10 ml of chloroform. Combine extracts in a separating funnel. Add 100 ml KOH solution, swirl gently for 30 sec and let layers separate. Place small plug of glass wool in 60 mm diameter filter funnel. Add about 25 g anhydrous Na_2SO_4. Drain lower chloroform layer through Na_2SO_4 into 25 ml glass stoppered graduated cylinder until 10 ml is collected (solution should be clear, if not refilter through small amount of Na_2SO_4).

Column chromatography: Silica gel is activated by heating in an oven at 110-130°C. Place a ball of glass wool at the bottom of 10 mm diameter glass column. Add about 0.5 g anhydrous Na_2SO_4 to give even base for silica gel. Add chloroform until column is about half full. Then slowly add 2.0 g of silica gel. Wash sides of column with chloroform and stir mixture with glass rod to remove air bubble and disperse silica gel. Slowly drain most of the chloroform leaving 2-3" above silica gel. Slowly add 0.5 g anhydrous Na_2SO_4. Wash column with 50 ml diethyl ether and then 10 ml chloroform to remove ether from column.

Mix 10 ml of sample extract with 30 ml hexane. Add this mixture to prepared column and drain at a rate at which distinct droplets are formed until solvent is about 1 cm above Na_2SO_4. Add in succession 30 ml benzene and 40 ml acetone-benzene (5 : 95) to column and let drain in same manner as above. Discard both solvents. Elute trichothecenes with diethyl ether until 30 ml is collected using same flow rate. Collect eluent in a glass stoppered graduated cylinder. Stopper graduated cylinder immediately so that, no ether evaporates (4 g sample in 30 ml diethyle ether).

Thin layer chromatography: Aliquots of T_2 extracts are then spotted over silica gel TLC plates. Plates are developed in toluene : ethyl acetate : formic acid (6 : 3 : 1, V/V). Fifty percent alcoholic H_2SO_4 is sprayed and dried at 110°C for 10 min. A sky blue fluorescent spot under UV indicate presence of T_2.

b. GLC method

Standard solutions for GLC: Prepare solutions of the following concentrations in ethyl acetate:

Solution 1 : 0.4 μg of T_2/ml
Solution 2 : 2.0 μg of T_2/ml

Internal standard solution : Prepare solutions of the following concentrations in pentane :

Solution 1 : 0.05 μg methoxychlor (MXCl)/ml
Solution 2 : 1.0 μg MXCl/ml

Internal standard factor : New internal standard factors (relative response of T_2 toxin to MXCl) should be obtained each day since response to GLC detector can vary from day to day.

1. Response factor solution 1 : Transfer 4.0 ml internal standard solution 1 to small tube. Add 2.0 ml working standard solution 1 and evaporate to dryness. Dissolve residue in 2.0 ml benzene by agitating on tube shaker about 30 sec. This solution contains 0.4 and 0.2 μg of T_2 and MXCl respectively.

2. Response factor solution 2 : Transfer 1.0 ml internal standard solution 2 and 1.0 ml working standard 2 to small tube, evaporate to dryness and dissolve residue in 2.0 ml benzene by agitating on tube shaker for about 30 sec. This solution contains 1.0 and 0.5 μg T_2 toxin and MXCl.

Derivatisation : Evaporate 15 ml diethyl ether eluent to dryness in small tube (save remaining diethyl ether fraction for confirmation purposes). Add 2 ml benzene (sample can be stored overnight at this point) and 100 μl heptafluorobutyrylimidazole (HFBI) derivatising reagent. Place teflon lined cap on the tube and mix solution vigorously on a mixer for 15 sec. Add about 1 ml 5% aqueous $NaHCO_3$ and shake on the mixer about 10 sec. Quantitatively transfer 0.5 ml benzene solution to third small tube, add 4.5 ml internal standard solution 1, place teflon lined cap on the tube and mix vigorously about 15 sec. Inject 5 μl of this solution into GLC.

For standards, derivatise, response factor solutions 1 and 2 by taking 2 ml of this solutions and add 100 μl HFBI. After the addition of $NaHCO_3$ take 0.5 ml of the benzene solution and add 4.5 ml of pentane. Inject 5 μl of this standard into GLC.

GLC conditions : Column 200, injector 205, detector 325 and interface 250°C. Carrier gas flow 24 ml/min. If column has not been used recently for T_2 toxin analysis remove the detector and inject 10 μl aliquots of silyl-8 and condition it overnight before use.

Calculation :

$$\text{Response factor} = \frac{\text{peak Ht MXCl / ng injected MXCl}}{\text{peak Ht } T_2 \text{ / ng injected } T_2}$$

, where, peak Ht= peak Ht of solution 2/peak Ht of solution 1 and ng injected MXCl or T_2 = ng of solution 2 / ng of solution 1.

If the amount of MXCl injected with sample extract is 0.225 ng and equivalent amount of sample injected is 0.005 g then,

$$T_2 \text{ in ppb} = \frac{0.225 \text{ ng MXCl x peak Ht } T_2 \text{ x response factor for } T_2}{\text{peak Ht MXCl x 0.005 g}}.$$

Note :

Sample should be analysed same day as derivatised.

References :

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2. J. Agric. Food Chem. 26, 647 (1978).

5. CLAVINE ALKALOIDS

Principle :

Alkaloids extracted on suitable medium and separated on TLC plates, when sprayed with Van Urk's reagent develops colour which can be read spectrophotometrically.

Reagents :

1. 0.1 N H_2SO_4
2. 95% H_2SO_4 (W/W)
3. 5% $FeCl_3$
4. Van Urk's reagent : Dissolve 0.125 g p-dimethylaminobenzaldehyde in 100 ml of 95% H_2SO_4 and add 0.1 ml of 5% $FeCl_3$.

Procedure :

Sample processing : Take 25 g of powdered ergot sclerotia, add 3 g of sodium bicarbonate and mix to a smooth paste with water. Extract with diethyl ether and further with tartaric acid. Lyophilise the acid extract. Dissolve in known quantity of chloroform. Apply aliquots of the extracted material on silica gel G coated TLC plates. Develop in chloroform : dimethylformamide : acetone : ethyl alcohol (6 : 2 : 1 : 1, V/V). The alkaloids can be detected by spraying Van Urk's reagent.

For chemical estimation, take 1 ml of the ergot extract in chloroform, dry at room temperature. Add 1 ml of 0.1 N H_2SO_4 and 2 ml of Van Urk's reagent and keep for 30 min with intermittent shaking. Measure the colour intensity in a Beckamn DU spectrophotometer at 550 and 720 nm. Calculations may be made by comparison with agroclavine standards.

References :

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XII. BACTERIOLOGY

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1. MICROSCOPIC EXAMINATION

Bacteria can be visualised under a microscope in saline suspensions directly or after staining. A series of such methods are described below.

a. Saline mount

Disperse a small quantity of the specimen to be examined into a drop of saline (0.85%) on a microscopic slide (3" x 1"). Overlay a coverslip and examine directly with a 40X or 100X objective of the microscope, lowering the condenser to reduce the amount of transmitted light.

This method can be used to determine the biological activity of microorganisms including motility, reactions to certain chemicals and serological reactivity in specific antisera. The latter includes quelling reaction (capsular swelling) used to identify different capsular types of *Streptococcus pneumoniae* and *Haemophilus influenzae*.

b. Hanging drop

A ring of modelling clay is placed on the slide. Cells from the bacterial colony to be examined are placed in the centre of the coverslip into a small drop of saline. The slide is inverted and pressed over the coverslips, guiding the drop of bacterial suspension into the centre of the well formed by the ring of modelling clay. The slide is carefully brought to an upright position for direct examination under the microscope. The edge of the drop is focussed where motility is best seen. This method is generally used for studying the motility of bacteria.

c. Iodine mount

Reagent:

Lugol's iodine solution: Dissolve 10 g of KI and 5 g of iodine crystals in 100 ml of distilled water.

Procedure:

A small amount of fecal matter is mixed in a drop of iodine solution on a microscope slide to form an even suspension and a coverslip is placed over the drop. Screen under low power for ova. Cysts are examined under high power.

This method is used in parallel with saline mounts for examining feces for intestinal protozoa or helminths ova. Iodine stains the nuclei and intracytoplasmic organelles. Iodine mounts should not be used to the exclusion of saline mounts, since iodine paralyses the motility of bacteria and protozoan trophozoites.

d. Gram's stain

This is one of the most commonly used stains in microbiology. It is a differential stain to demonstrate the staining properties of all types of bacteria.

Reagents:

1. Crystal violet solution: Dissolve 2 g of crystal violet in 20 ml of 95% alcohol and 0.8 g of ammonium oxalate. Make up the volume to 100 ml with distilled water.
2. Gram's iodine solution: Dissolve 2 g of KI and 1 g of iodine crystals in 100 ml distilled water.
3. Decolorising solution: Mix 50 ml of acetone and 50 ml of 95% alcohol.
4. Counterstain solution: Dissolve 2.5 g of safranin-O in 100 ml of 95% alcohol. Take 10 ml of this and dilute with 100 ml of distilled water.

Procedure:

Make a thin smear of the material under study and allow to air dry. Fix the material to the slide so that it does not wash off during the staining procedure by passing the slide 3 or 4 times through the flame of a Bunsen burner.

Place the smear on a staining rack and overlay the surface with crystal violet solution. After 1 min of exposure to the crystal violet stain, wash thoroughly with distilled water. Next, overlay the smear with Gram's iodine solution for 1 min. Again wash with water. Flood the smear with a few drops of decolorising solution until no violet colour washes off. This usually requires 10 sec or less. Wash with running tap water. Overlay the surface with safranin counterstain for 1 min. Wash with running water. Place the smear in a upright position in a staining rack allowing the excess water to drain off and the smear to dry.

Examine the smear under the 100X (oil immersion) objective of the microscope.

The gram positive bacteria retain the crystal violet dye after decolorisation and appear deep blue.

Gram negative bacteria are not capable of retaining the crystal violet dye after decolorisation and are counterstained red by the safranin dye.

Gram staining characteristics may be atypical in very young, old, dead or degenerating cultures.

e. Special stain

Special staining techniques are used to visualise organisms like mycobacteria which do not take up ordinary stain or to differentially stain diphtheria bacilli.

(i) Ziehl-Neelsen stain for tubercle bacilli

Principle:

Ordinary aniline dye solutions do not readily penetrate the tubercle bacilli. A heated staining solution containing phenol can be made to penetrate the bacteria. Once stained these organisms resist decolorisation.

Reagents:

1. Carbol fuchsin : Dissolve basic fuchsin 10 g in absolute alcohol 100 ml and add 1 l of 5% phenol solution.
2. 20% Sulphuric acid
3. 95% Alcohol
4. Loffler's methylene blue : Mix, 300 ml of saturated solution of methylene blue in alcohol, in 1 l of 0.01% KOH.

Procedure:

Cover the slide with heated carbol fuchsin and heat till steam rises. Keep for 5 min applying heat at intervals to keep the stain hot. If the stain begins to dry, pour more carbol fuchsin. Wash with water. Cover with 20% sulphuric acid and keep for 1 min. The red colour of the preparation becomes yellowish brown. Wash with water and pour more acid. Repeat this process several times till the slide has a faint pink colour. Decolorisation generally requires contact with sulphuric acid for a total time of 10 min. Wash with water and keep in 95% alcohol for 2 min. Wash with water. Counterstain with Loffler's methylene blue for 15-20 sec. Wash with water and air dry.

Acid fast bacilli stain bright red, while the tissue cells and other organisms are stained blue. If the tissue cells appear red, the preparation has not been adequately decolorised.

(ii) Albert-Laybourn stain for diphtheria bacilli

Reagents:

1. Albert's stain : Dissolve toludine blue 1.5 g and malachite green 2.0 g in 20 ml of 95% alcohol and add to 1 l water containing 10 ml of glacial acetic acid.
2. Albert's iodine : Dissolve 9 g of potassium iodide in 250 ml water. When dissolved add 6 g of iodine and make up to 900 ml with distilled water.

Procedure:

Cover the slide with Albert's stain. Keep for 3-5 min. Wash with water and pour Albert's iodine. Keep for 1 min. Wash with water and air dry.

The metachromatic granules of diphtheria bacilli stain bluish black, the protoplasm green and other organisms light green.

2. CULTURE EXAMINATION

a. Preparation of culture

This process involves three distinct steps :

1. Collection of sample : Samples for culture should be collected before starting antimicrobial therapy. The specimens should be collected in sterile containers and transported immediately to the laboratory. When a delay is anticipated specimens should be collected in appropriate transport media.

2. Selection of culture media : There are two types of media, viz. liquid and solid. In the liquid media, characteristic appearance of a particular bacterium cannot be seen and further it cannot be separated from other coexisting bacteria. They are however useful when designed for a particular biochemical test. The liquid media are made solid by the addition of agar. This makes the media useful for the identification and isolation of pure culture from mixtures.

3. Sterilisation : As has been emphasised in the collection of the sample, it is absolutely essential that the media, the containers and the samples are free of all bacteria other than that in the specimen.

b. Preparation of common media

1. Glycerol-saline transport medium : Dissolve 4.2 g of NaCl in water and add 300 ml of glycerol. To this is added 10 g of anhydrous Na_2HPO_4 and dissolve it by heating. Add 15 ml of 0.02% aqueous phenol red and make upto 700 ml with water.

Distribute in 6 ml aliquots in universal containers and autoclave at 115°C for 20 min (The solution should be pink in colour and if it turns yellow it should be discarded).

2. Venkatraman-Ramakrishnan fluid : Dissolve 3.1 g of boric acid and 10.5 g KCl in 20 ml of hot water. Cool and make up the volume to 250 ml. Add 133.5 ml of 0.2 M NaOH and make up the volume to 1 l. Dissolve 180 g of NaCl, 20 g of $MgCl_2$ and 11.7 g of $MgSO_4$. Filter and distribute in 10 ml aliquots in screw capped bottles and autoclave at 121°C for 20 min.

3. Deoxycholate-citrate- agar medium :

A. Neutral red-lactose-agar solution : Dissolve 20 g of beef extract in 100 ml water by heating. Make just alkaline to phenolphthalien with 5.0% NaOH, boil at 100°C and filter. Adjust the pH to 7.4, make upto 200 ml and add 20 g of peptone. Dissolve 90 g of agar in 3.8 l water by steaming for 1 hr. Mix beef extract peptone solution and add 5 ml of neutral red solution (2% in 50% ethanol and 40 g of lactose, bottle in 100 ml lots and autoclave at 115°C for 15 min.

B. Citrate solution : Dissolve 17 g each of sodium citrate and sodium thiosulfate and 4 g of ferric ammonium citrate in 100 ml distilled water by heating it at 60°C for 1 hr.

C. Deoxycholate solution : Dissolve 10 g of deoxychlolate in 100 ml sterile water.

Dissolve by heating 100 ml of reagent A and add 5 ml each of reagents B and C. Pour onto sterile plates (the medium should be pale pink in colour).

4. MacConkey's agar medium : Dissolve 20 g peptone and 5 g taurocholate in minimum amount of water by heating. Adjust the pH to 7.5. Add 20 g agar, 100 ml of 10% lactose solution and 3.5 ml neutral red solution (2% in 50% ethanol). Autoclave at 115°C for 15 min. Pour into sterile plates (the medium should be distinct reddish brown in colour).

5. Selenite F broth : Dissolve 4 g sodium acid selenite, 5 g peptone, 4 g lactose, 9.5 g Na_2HPO_4 in 1 l of sterile water. Distribute in 10 ml aliquots in screw cap tubes. Steam at 100°C for 30 min. (Do not autoclave, pH should be 7.1).

6. Thiosulfate-citrate-bile-sucrose-agar medium (TCBS) : Dissolve peptone 10 g, yeast extract 5 g, sodium taurocholate 5 g, sodium thiosulfate 10 g, NaCl 20 g, sodium lauryl sulfate 0.2 g and sodium citrate 10 g in 200 ml of water and adjust pH to 8.6. Add agar 20 g, sucrose 20 g, bromothymol blue 0.04 g and thymol blue 0.04 g and make upto 1 l with water. Steam for 1 hr and then autoclave at 115°C for 15 min.

7. Alkaline-peptone water : Dissolve peptone 10 g and NaCl 5 g in 1 l distilled water. Adjust the pH to 8.4. Autoclave at 121°C for 15 min.

8. Blood-agar solution : Dissolve beef extract 10 g, peptone 10 g and NaCl 5 g in distilled water. Adjust pH to 7.4. Add 20 g agar, make upto 1 l with water and autoclave at 121°C for 15 min. Cool to 55°C and then add 50 ml of sterile sheep blood. Pour onto sterile plates immediately.

c. Stool culture

The sample should be collected in wide mouthed sterile container with glycerol-saline transport medium for *Salmonella* and *Shigella* and Venkatraman fluid for *Vibrio*

Inoculate the sample onto (1) DCA, (2) MacConkey and (3) Selenite F broth. Incubate at 37°C overnight.

Examine the plates for non-lactose fermenting (NLF) colonies. Pick up the NLFs and identify by biochemical reactions (see the Tables) and slide agglutination tests.

Subculture from selenite F broth on DCA and MacConkey after 18 hr. Examine and identify any NLFs present.

If *Vibrio cholera* is suspected, inoculate into alkaline peptone water and TCBS medium. Incubate the alkaline peptone water for 6 hr at 37°C. Subculture on TCBS and incubate the plates at 37°C overnight. Yellow colonies of *Vibrio cholera* should be identified by biochemical tests and serology.

d. Urine culture

Mid stream urine sample should be collected in a sterile container and sent to the laboratory immediately. The time between the collection of the sample and plating should not exceed 4 hr during which time it should be stored in the refrigerator.

Inoculate with a standard loop (4 mm internal diameter delivering 0.01 ml) on (1) blood agar and (2) MacConkey's medium. Incubate at 37°C overnight. Count and identify any organisms present.

Presence of more than two types of organisms in small numbers indicates contamination. More than 10^5 organisms per ml indicates infection. Test the organism for antimicrobial sensitivity using antibiotic impregnated filter paper discs.

References :

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TABLES

Identification of vibrous and allied organisms

Organism	TSI butt			Oxidase	Lysine	Mannitol
	Acid	Gas	H_2S			
Vibrio	+	—	—	+	—	+
Aeromonas	+	d	—	+	+	+
Plesiomonas	+	—	—	+	+	—
Enterobacteriaceae	+	d	d	—	d	d

+ = positive ; — = negative: d = variable or different reaction.

Differentiating charecters of the main genera of enterobacteriaceae and some exceptional specics

Genus	Acid from lactose	Gas from glucose	Motility	Urease	Citrate utilised*	Voges-Proskauer	Growth in KCN
Escherichia	+	+	+	−	−	−	−
Shigella	−	−	−	−	−	−	−
Citrobacter	+	+	+	d	+	−	+
Salmonella	−	+	+	−	+	−	−
Enterobacter	+	+	+	d	+	+	+
Hafnia	−	+	+	−	+	d	+
Klebsiella	+	+	−	+	+	+	+
Serratia	d	d	+	−	+	+	+
Proteus	−	+	+	+	d	−	+
Providencia	−	+	+	−	+	−	+

* Citrate utilisation tested in Kosre's or Simmons's medium.
+ = most or all strains postitive.
− = most or all strains negative.
d = some strains positive, some negative.

Reaction	*Escherichia coli*	A–D Group	*Klebsiella aerogenes*	*Sh. dysenteriae*	*Sh. flexneri*	*Sh. oydii*	*Sh. sonnei*	*Enterobacter cloacae*	*Hafnia alvei*	*Serratia marcescens*	*Citrobacter*	*Proteus vulgaris*	*Proteus mirabilis*	*Proteus morganii*	*Proteus rettgeri*	*Providencia*
Motility	+	−	−	−	−	−	−	+	+*	+	+	+	+	+	+	+
Capsule	−	−	+	−	−	−	−	−	−	d	−	−	−	−	−	−
Glucose (gas)	+	−	+	−	−	−	−	+	+*	d	+	+	+	+	−	d
Lactose (acid)	+	d	+	−	d	−	(+)	+	−	(±)	d	−	−	−	−	−
Sucrose (acid)	d	d	+	−	−	−	(+)	+	d	+	d	+	(+)	−	(+)	(+)
Dulcitol (acid)	d	d	d	−	d	d	−	−	−	−	d	−	−	−	−	−
Inositol (acid)	−	−	+					d	−	d	−	−	−	−	+	d
Mannitol (acid)	+	+	+	−	+	+	+	+	+	+	+	−	−	−	+	d
Indole	+	+	−	−	−	d	−	−	−	−	d	+	−	+	+	+
Methyl Red	+	+	−	+	+	+	+	−	−*	−	+	d	..	..	..	+
Voges-Proskauer	−	−	+	−	−	−	−	+	+*	+	−	−	d	−	−	−
Citrate	−	−	+	−	−	−	−	+	+*	+	+	d	d	−	+	+
Gelatin	−	−	−	−	−	−	−	(+)	−	+	−	+	+	−	−	−
Phenylalanine	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
Urease	−	−	+	−	−	−	−	d	−	−	d	+	+	+	+	−
Hydrogen sulphide	−	−	−	−	−	−	−	−	+	−	+	+	+	−	−	−
KCN	−	−	+	−	−	−	−	+	+	+	+	+	+	+	+	.

+ = Result positive with at least 80% of strains.
− = Result negative with at least 80% of strains.
d = Different result in different strains.
(+) = Delayed positive result (e.g.2–8 days).
.. = Result not given.
* tests done at 25°–30°C.

XIII. CYTOGENETICS

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1. CHROMOSOME PREPARATION

a. Lymphocytes

Principle :

Since peripheral lymphocytes are not spontaneously dividing cells, there is a need to stimulate their division *in vitro* by adding a mitogen. Phytohemagglutinin is the most commonly used one. The dividing cells are to be accumulated at metaphase by treating cultures with colchicine or its derivatives. The cells are collected, allowed to swell by hypotonic treatment. This promotes dispersion of metaphase chromosomes.

Reagents :

1. Stock TC 199 medium : The powder is dissolved in 100 ml sterile glass distilled water and stored at 4°C.

2. Antibiotic solution : A mixture of 0.5 g streptomycin and 5 lakh units penicillin is prepared in 1 l of sterile water.

3. Phytohemagglutinin : The contents of one vial is dissolved in 5 ml sterile glass distilled water and stored at 4°C.

4. Working medium : Mix 10 ml stock TC 199 medium, 90 ml sterile glass distilled water, 25 ml fetal calf serum and 1 ml of antibiotic solution. The pH of the solution is adjusted to around 7.2.

5. 0.075 M KCl.

6. Colchicine : 2.5 mg in 100 ml water.

7. Giemsa stain : Mix 5 ml of Giemsa stain, 95 ml of distilled water and 2 ml of 0.15 N NH_4OH. Prepare fresh.

Procedure :

Each culture tube should contain 5 ml of working TC 199 medium, 0.1 ml of phytohemagglutinin solution and 0.4 ml heparinised whole blood. Incubate the tubes at 37°C for 69 hr, add 3 drops of colchicine to each tube and continue the incubation for 3 more hours.

The tubes are centrifuged for 5 min at 800 rpm. The supernatant is discarded and the cells are treated with 5 ml KCl solution for 7 min. The suspension is again centrifuged and the supernatant is removed completely. The cells are then fixed in 3 : 1 methanol-acetic acid mixture. Store at 4°C for 15-20 min. Repeat washing for 3 more times avoiding further fixation at 4°C. The cells are finally suspended in a suitable volume of fixative and one drop of solution is dropped onto chilled slide. The slides are air dried and stained with Giemsa for 15 min.

References :

1. Exptl. Cell Res. **20**, 613 (1960).
2. Human Chromosome Methodology, J. J. Yunis (Ed.). Academic Press, 2nd Edn. p. 95 (1974).

b. Bone marrow

Principle :

Bone marrow contains large number of dividing cells for direct chromosome analysis unlike lymphocytes.

Reagents :

1. 0.7% Sodium citrate
2. 0.075 M KCl
3. Methanol-acetic acid (V/V) 3 : 1

Procedure :

Two hr prior to sacrifice, animal (rat or mouse) is administered intraperitoneally colchicine (0 7 mg/Kg body weight). Bone marrow from femora is aspirated into Hank's balanced salt solution. The solution is aspirated through a syring-needle 2 or 3 times to obtain a fine cell suspension. The solution is centrifuged for 5 min at 600 rpm. The supernatant is discarded and the cells are treated with 6 ml hypotonic solution (sodium citrate for rats and KCl for mouse) for 12-15 min. The suspension is again centrifuged and the supernatant is removed. The cells are then fixed in 3 : 1 methanol-acetic acid. The process is repeated thrice with an interval of 15 min between first and second treatment when the tubes are kept at 4°C. Prepare slides by air drying and stain it with Giemsa.

Referenec :

Human Chromosome Methodology, J.J. Yunis (Ed.) Academic Press, 2nd Edn. p. 157 (1974).

2. SISTER CHROMATID EXCHANGES

a. Human and monkey lymphocytes

Principle :

Chromosomes of cells which have undergone two rounds of DNA synthesis in the presence of 5-bromodeoxyuridine (BUdr) exhibit differential chromatid staining when stained with a photosensitive dye Giemsa system. Sister chromatid exchanges (SCEs) are apparent as sharp reciprocal alterations along the chromosomes.

Reagents :

1. Culture medium : Dissolve 1 g RPMI - 1640 in 77 ml glass distilled water, sterilise through millipore filter and add sterile solutions of fetal calf serum 20 ml, antibiotics 1 ml (Refer Chromosome preparation), 2.92% L - glutamine 1 ml and PHA 1 ml. Adjust pH to 7.2.

2. BUdr solution : Dissolve 30.76 mg of BUdr in 100 ml glass distilled water, sterilise through millipore filter.

3 Dye solution : 0.5 μg/ml of Hoechst 33258 dye. This is kept at 4°C in the dark at 100 X concentration.

Procedure :

Take 0.1 ml of BUdr solution, 10 ml of culture medium and add 0.8 ml of whole blood. Incubate for 72 hr at 37°C in the dark, add colchicine at 69th hr. Harvest as for normal blood chromosomal cultures.

Stain for 20 min with Hoechst dye solution. Mount after placing a drop of phosphate buffer (pH 7.2), seal the edges with rubber solution. Expose to fluorescent day light lamp for 6-10 hr when kept at a distance of 5 cm from light. If sunlight is used expose the slides under these conditions for 2 hr.

Remove coverslips, incubate in 2 X SSC at 60°C for 20–30 min. Stain in 4% Giemsa for 8–10 min. Rinse in water, dry and mount in DEPEX, score for the frequency of SCEs per chromosome.

b. Rabbit lymphocytes

Procedure :

Incubate 10 ml of RPMI - 1640 medium, 0.8 ml whole blood and 0.1 ml of PHA at 39°C in a water bath for 20-22 hr. After this period remove the old medium without loosing cells. Place fresh medium without PHA, add BUdr to give a final concentration of 10 μM and incubate cultures for 30 more hours in the dark at 39°C. Prepare chromosomes (Refer Chromosome preparation) and stain for SCE as for human and monkey chromosomes.

Reference :

Mutation Res. **41**, 333 (1976).

c. Rat and mouse bone marrow

Principle :

In vivo analysis of SCEs in rat and mouse bone marrow is done by keeping a sufficient concentration of BUdr for DNA labelling over one synthetic cycle. This is achieved by the use of serial injections of BUdr. In addition, application of 5-fluorodeoxyuridine (FUdr) prior to the administration of BUdr provides effective labelling of DNA.

Procedure :

Administer intraperitoneally 0.4 mg/g body weight of FUdr to animals. Three hr later give BUdr

(0.04 mg/g body weight) for six times at hourly interval. After 27 hr following the initial injection of FUdr, give colchicine (0.7 mg/Kg body weight). Prepare bone marrow chromosomes 3 hr later, and stain for SCE as described earlier.

Reference:

Nature **260**, 448 (1976).

XIV. HISTOPATHOLOGY

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1. PROCESSING OF TISSUES

a. Fixation

Principle :

Fixation is the process of preserving, hardening and preventing postmortem changes of the tissues. Tissue should be placed in the fixative immediately after removal from the body. Tissue blocks are cut of such thickness (usually about 3 mm) that the fixative readily penetrates throughout the tissue in a reasonably short time. Volume of fixative employed is 15-20 times that of the tissue to be fixed.

Commonly used fixatives :

1. 10% Neutral buffered formaldehyde solution, pH 7.0 : Mix 100 ml of 37-40% formaldehyde, 900 ml of water, 4 g of sodium phosphate monobasic and 6.5 g of sodium phosphate dibasic.

2. Zenker's fluid, stock solution : Dissolve mercuric chloride 50 g, potassium dichromate 25 g and sodium sulphate 10 g in 1 l of water.

5 ml of glacial acetic acid is added to 95 ml of Zenker's stock fluid before use.

3. Zenker-formalin solution (Helly's) : Prepare by adding 5 ml of 37-40% formaldehyde instead of acetic acid to 95 ml of Zenker's stock solution.

4. Bouin's solution : Mix 750 ml of saturated picric acid with 250 ml of 37-40% formaldehyde and 50 ml of glacial acetic acid.

Procedure:

The most commonly used fixative is 10% formalin since it is compatible with most stains. The duration of fixation depends on the size of the tissue blocks. Generally, 24 hr is adequate. Zenker's fluid is used for fixing muscle and testis. Bouin's solution is used for fixing testicular biopsies and for the differential staining of connective tissues. Tissue block should not be more than 5 mm in thickness and fixed for 6-18 hr. They are then washed in running water overnight and stored in 70% alcohol.

b. Tissue processing

Principle:

This involves dehydration, clearing and infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue is transferred to a paraffin solvent which is miscible with the dehydrating agent as well. These are known as clearing agents eg. chloroform, xylene etc.

Procedure:

A specimen brought to the laboratory is marked with an identifying number. This number is kept with the tissue block throughout processing. The tissue block is now conveyed through a series of following solvents as per the schedule for dehydration, clearing and paraffin infiltration.

Alcohol 80%	—	1 hr
Alcohol 90%	—	1 hr
Alcohol 95% -2 changes	—	1 hr each
Isopropyl alcohol	—	1 hr
Acetone - 2 changes	—	1 hr each
Chloroform - 3 changes	—	1 hr each
Paraffin - 2 changes	—	3 hr each

This block is then ready for embedding.

During the process of embedding, the tissue blocks are oriented so that sections are cut in the desired plane of the tissue. Two L-shaped metal moulds are laid on metal plate so as to enclose a rectangular or square space. This is than partly filled with melted paraffin and the tissue is placed in it in the desired position. The container is then filled with melted paraffin and allowed to cool until reasonably firm so that the set block of paraffin with the tissue can be removed from the moulds. The block is then trimmed to a suitable size and fixed on a metal object holder. The block is further trimmed so that paraffin overlying the piece of tissue is excluded and an adequate area of the tissue facing the knife is exposed. The block is then kept for cooling at 0°C.

c. Section cutting

Usually sections are cut at 5μm thickness and floated in a water bath between 38-49°C. The sections from the water are mounted on clean glass slides which have been smeared with a drop of Mayer's egg albumin. They are then dried on a hot plate at about 50°C for 30 min. The sections on the slides are then ready for staining.

d. Staining procedures (Hematoxylin and Eosin)

Reagents:

1. Mayer's Hematoxylin stain : Dissolve 50 g of ammonium or potassium alum in 1 l of water without heating. Then dissolve hematoxylin 1 g in this solution. Further add 0.2 g sodium

iodate, 1 g citric acid and 50 g chloral hydrate. Shake until all components are in solution. The final colour of the stain is reddish violet. Stain keeps well for a month.

2. 1% Stock Eosin solution : Dissolve 1 g of Eosin Y (water soluble) in 20 ml distilled water and make up to 100 ml with 95% alcohol.

Working Eosin solution : Dilute 1 part of the stock solution with 3 parts of 80% alcohol. Add 0.5 ml of glacial acetic acid for every 100 ml of stain.

Procedure :

The slide containing the section is processed serially as follows :

1. Xylol I	—	3 min
2. Xylol II	—	3 min
3. Acetone	—	3 min
4. 95% Alcohol	—	3 min
5. Running water	—	3 min
6. Hematoxylin stain	—	20 min
7. Wash in running tap water	—	20 min
8. Eosin working solution	—	15 sec-2 min
9. 95% Alcohol	—	2 to 3 dips
10. 95% Alcohol – 2 changes	—	1 to 2 min each
11. Acetone - 2 changes	—	3 min each
12. Xylol - 2 changes	—	3 min each

13. Mount in D. P. X. and view it under microscope.

The nuclei are stained blue and cytoplasm in various shades of pink.

2. SPECIAL STAINS

a. Alcian blue - periodic acid Schiff stain

Reagents:

1. Alcian blue stain: Dissolve 1.0 g of alcian blue in 100 ml of 3% acetic acid. The solution is filtered and crystals of thymol are added to preserve. The pH should be about 2.7 (2.5 - 3.0). The solution remains stable for 2 - 3 weeks. It is filtered, if necessary, before using.

2. Periodic acid solution : Dissolve 0.5 g of periodic acid in 100 ml of distilled water.

3. Schiff's reagent : 1 g of basic fuchsin is dissolved in 200 ml of hot (90-95°C) distilled water and filtered at 50-60°C as it cools. Cool and add 2 g of $NaHSO_3$ followed by 20 ml 1 N HCl, stopper tightly and store overnight in the dark, at room temperature. Powdered charcoal 500 mg is then added, shaken for 1 min and filtered twice. The solution should be clear. It is stored in an amber coloured bottle at 5°C. If kept at this temperature, it remains active for some months. When any pink tint appears, it should be discarded.

4. Reducing rinse solution, stock : Dissolve 10.4 g of $NaHSO_3$ in 100 ml of water.

Working solution : Dilute 1 part of stock solution with 20 parts of distilled water.

Procedure :

Follow the following sequence of events for staining :

1.	Xylol I, Xylol II, Acetone, 95% Alcohol, Distilled water - As in the case of Hematoxylin stain		
2.	Alcian blue	—	30 min
3.	Wash in running tap water	—	2 min
4.	Distilled water	—	1 dip
5.	0.5% Periodic acid	—	10 min
6.	Wash in running tap water	—	5 min
7.	Distilled water	—	1 dip
8.	Schiff's reagent	—	10 – 15 min
9.	Reducing rinse solution-3 changes	—	2 min each
10.	Wash in running tap water	—	5 min
11.	Hematoxylin stain	—	5 min
12.	Wash in running tap water	—	2 min
13.	95% Alcohol followed by absolute alcohol	—	2–3 dips
14.	Xylol–2 changes	—	3 min each
15.	Mount in Canada Balsam or D. P. X.		

Acid mucopolysaccharides stain blue, glycogen and basement membrane stain pink and nuclei stain purple.

Note :

If Zenker-fixed, the mercury deposits are removed with iodine and sodium thiosulfate. Staining with alum Hematoxylin can be omitted when nuclear stain is not desired.

b. Gomori's reticulum stain

Reagents :

1. Silver oxide solution : 4 ml of 10% KOH is added to 20 ml of 10% silver nitrate. Ammonia water 28% is then added drop by drop while shaking the container continuously until the precipitate is completely dissolved. Silver nitrate 10% solution is again added but cautiously drop by drop until the resulting precipitate disappears easily by shaking the solution. The solution is then diluted with an equal volume of distilled water.

2. Potassium permanganate solution : Dissolve 0.5 g of potassium permanganate in 100 ml of distilled water.

3. Oxalic acid solution : Dissolve 5 g of oxalic acid in 100 ml of distilled water.

4. Ferric ammonium sulfate solution : Dissolve 2.0 g of ferric ammonium sulfate in 100 ml of distilled water.

5. Formalin solution : 10 to 20%.

6. Gold chloride solution : Dissolve 0.2 g of gold chloride in 100 ml of distilled water.

7. Potassium metabisulfite solution : Disslove 1 g of potassium metabisulfite in 100 ml of water.

8. Sodium thiosulfate solution : Dissolve 1 g of sodium thiosulfate in 100 ml of water.

Procedure :

Follow the following sequence for staining :

1.	Xylol I, Xylol II, Acetone, 95% Alcohol and Distilled water	—	As in the case of Hematoxylin
2.	Potassium permanganate solution	—	2 min
3.	Rinse in tap water	—	2 min
4.	Oxalic acid solution	—	2 min
5.	Rinse in tap water	—	2 min
6.	Ferric ammonium sulfate solution	—	1 min
7.	Rinse in tap water	—	2 min
8.	Distilled water–2 changes	—	3 dips each
9.	Silver oxide solution	—	1 min
10.	Distilled water	—	5–10 sec
11.	10 or 20% Formalin	—	3 min
12.	Rinse in tap water	—	3 min
13.	Gold chloride solution	—	10 min
14.	Distilled water	—	2–3 dips
15.	Potassium metabisulfite solution	—	1 min
16.	Sodium thiosulfate	—	1 min
17.	Rinse in tap water	—	3 min
18.	Absolute alcohol–2 changes	—	2 min each
19.	Xylol–2 changes	—	3 min each

20. Mount in Canada Balsam and observe under microscope.

The reticulum fibers are stained black and background grey.

c. Masson's trichrome stain

Reagents:

1. Bouin's solution : Refer Fixation.
2. Weigert's iron Hematoxylin solution :
   Solution A : Dissolve 1 g of Hematoxylin crystals in 100 ml of 95% alcohol.
   Solution B : Mix 4 ml of 29% ferric chloride, 1 ml of conc. HCl and 95 ml of water.
   To prepare a working solution, mix equal parts of A and B solutions.

3. Biebrich scarlet-acid fuchsin : Mix 90 ml of 1% aqueous Biebrich scarlet, 10 ml 1% aqueous acid fuchsin and 1 ml of glacial acetic acid.

4. Phosphomolybdic-phosphotungstic acid solution : Dissolve 5 g of phosphomolybdic acid and 5 g of phosphotungstic acid in 200 ml distilled water.

5. Aniline blue solution : Dissolve 2.5 g of aniline blue in 2 ml of glacial acetic aicd and 100 ml distilled water.

6. 1% Glacial acetic acid : Mix 1 ml of glacial acetic acid with 100 ml of distilled water.

Procedure :

Follow the following sequence for staining :

1.	Xylol I, Xylol II, Acetone, 95% Alcohol and Distilled water	—	As in the case of Hematoxylin
2.	Bouin's fluid at 56°C	—	1 hr or overnight at room temperature if formalin fixed
3.	Cool, rinse in running tap water	—	10 min
4.	Distilled water	—	3 dips
5.	Weigert's iron Hematoxylin	—	10 min
6.	Rinse in running tap water	—	10 min
7.	Distilled water	—	3 min
8.	Biebrich scarlet-acid fuchsin	—	2 min (save the solution)
9.	Distilled water	—	3 min
10.	Phosphomolybdic-phosphotungstic acid	—	10–15 min
11.	Aniline blue	—	5 min (save the solution)
12.	Distilled water	—	3 min
13.	Acetic acid solution	—	3 min
14.	95% Alcohol 2 changes	—	1 dip each
15.	Acetone 2 changes	—	3 min each
16.	Xylol–2 changes	—	3 min each
17.	Mount in D.P.X. and view it under microscope.		

The nuclei are stained black, cytoplasm, keratin, muscle and intercellular fibers are stained red and collagen blue.

3. FROZEN SECTION

a. Preparation of section

Principle :

Cold microtome (cryostat) is often used for section cutting. Frozen sections are convenient for rapid diagnosis as a routine and necessary for enzymatic and non-enzymatic histochemical studies.

Procedure :

Tissue blocks (2-4 mm) with or without prior fixation are frozen in a mixture of dry ice and acetone or in isopentane cooled by liquid nitrogen. They are cut immediately in the cryostat at the desired thickness often ranging between 5-15 μm. The tissues meant for enzymatic histochemical studies should be frozen and cut immediately after they are removed from the animal or human subject (by way of biopsy or autopsy), without any prior fixation. The sections are picked up from the knife on to coverglass or slides or received directly into the stain, incubating medium or distilled water whichever may be necessary. The sections of the unfixed material after having been received onto coverglass or slides may be placed in a suitable fixative for non-enzymatic histochemical studies. For similar studies the tissue blocks prior fixed in 10% formalin can also be employed.

b. Enzyme histochemistry

The frozen sections mounted on a slide or without mounting can be used for the identification and localisation of an enzyme in a tissue. This is achieved by incubating the sections in an appropriate buffer containing the substrate. The product formed is localised *in situ* and has to be converted to a substance which is visible under microscope. Those reactions where oxido-reduction is involved, substances like tetrazolium blue is used. They form formazan which are insoluble and are coloured. On the otherhand where the end product is an inorganic ion like phosphorus, it is made insoluble initially with calcium chloride and made into a coloured complex by treating with cobalt chloride and ammonium sulphide which gives an insoluble cobalt sulfide-black in colour.

c. Non-enzyme histochemistry

Principle:

Unlike enzyme histochemistry, it is the process of *in situ* staining of the tissue constituents to localise and identify them. For eg. for demonstrating fat in tissues the following procedure may be adopted.

Reagent :

Stock saturated solution of oil red-O : Dissolve 250-500 mg of oil red-O in 100 ml of isopropyl alcohol. The working solution is prepared by mixing 6.0 ml of the stock solution with 4.0 ml distilled water. The solution is stirred for 5-10 min and then filtered. The filtrate can be used for several hours.

Procedure :

Frozen sections (10-15 μm) are received directly into oil red-O or in distilled water and then transferred to oil red-O solution. They are allowed to remain in the stain for 10 min. (Staining of floating sections gives better results than the sections mounted on coverglass or slide) Wash it in running water and stain it with acid alum Hematoxylin for 5 min. Trensfer this to tap water, so that they may turn blue. The sections are floated in water and then taken on slides. The water is drained off and mounted in gum syrup. The fat globules are stained red.

4. DECALCIFICATION

Principle :

Calcification is the term applied to organic tissues which have been infiltrated with calcium salts. These salts must be removed to assure that the specimen is soft enough to allow section cutting.

Reagents :

1. Mix 150 ml each of concentrated HCl and HNO_3 in 2.7 l of 80% alcohol
2. 10% EDTA (dipotassium salt)
3. 5% Ammonium hydroxide
4. 5% Ammonium oxalate

Procedure :

Bone and other calcified material should be cut into small pieces with fine saw. After sufficient fixation the pieces are placed in a large amount of decalcifying solution (either reagent 1 or 2). Stirring

and heating hastens decalcification. Tissues should be suspended in the upper 1/3 of fluid during decalcification, so that calcium salts sink to the bottom of the container. Since the decalcifying solution contains acid it should be borne in mind that the tissues are washed thoroughly to remove acid before subsequent processing.

It is very important to determine the end point to remove the tissues from the decalcifying fluid once the decalcification is accomplished. If this is not done, the chances of subsequent good staining reactions are reduced by 10% for every 2 hr the tissue remains in the decalcifying fluids. For this draw approximately 5 ml of decalcifying fluid (from the bottom of container) which has been in contact with tissue 6–12 hr. Add 5 ml each of 5% ammonium hydroxide and ammonium oxalate. Mix and let it stand 15–30 min. A cloudy solution is formed by calcium oxalate indicates that the specimen is not thoroughly decalcified. Change the decalcifying solution and perform the test at a later time. When a milky solution is no longer obtained from such a mixture, the specimen is completely decalcified.

Note :

The tissues should not be left in the decalcifying solution overnight eventhough the decalcification is incomplete. Return to formalin and continue the process next morning.

References :

1. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. L.G. Luna (Ed.), 3rd Edn. McGraw – Hill Book Company (1968).

2. Lynch's Medical Laboratory Technology. S. S. Raphael (Ed.) 3rd Asian Edn. W. B. Saunders Company, p. 934 (1976).

XV. HAEMATOLOGICAL TECHNIQUES

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1. ENUMERATION OF RED BLOOD CORPUSCLES

Principle:

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with the blood and which will prevent its coagulation. A dilution of 1 to 200 is usually necessary. The diluted blood is placed in a counting chamber and the number of cells in a circumscribed volume is enumerated under a microscope.

Materials:

1. Red cell pipette : It is a capillary tube, graduated in 10 ths, which opens into a bulb, with a red glass bead. The bulb when filled to the mark above it (101) will hold 100 times the quantity of fluid contained in 10 divisions of the capillary tube.

2. Counting chamber : A Neubauer counting chamber (hacmoeytometer) with ratings is commonly used.

3. Diluting fluid : A solution of 1% formalin (10 ml/l, 40% formaldehyde) in 31.3 g/l trisodium citrate.

Procedure:

The blood is drawn by mouth suction upto the 0.5 mark, the tip of the pipette is wiped clean and the diluent is drawn in until the solution fills the pipette and reaches the '101' mark. If the blood is being taken directly from the finger of the subject or the tail of a rat, this procedure should be done quickly to avoid coagulation. The pipette is shaken by holding it loosely in one hand (after removing the attached rubber tubing) between the thumb and the forefingers at least for 3 min before loading it in the chamber. Alternately, 20 μl of blood can be diluted in 4.0 ml of the diluting fluid in a test tube.

The coverslip is put on the counting chamber. A few large drops of the mixed solution are discarded from the pipette and then a small quantity of the diluted blood is put between the coverslip and the ruled platform of the counting chamber. The chamber should not overflow and there should not be any air bubble in the chamber. The solution is allowed to settle for a couple of minutes and then the counting is done under the high power of a microscope.

In the Neubauer ruling, the small squares in the central large 1 mm square are used for the enumeration of erythrocytes.

The number of cells in the 4 corner groups of 16 squares are counted and also one central group including those cells which lie within the area or on the dividing lines to the left or above the section. If the dilution has been 1 to 200 (blood drawn to 0.5 mark) then the total number of cells found in the 5 groups of 16 squares is multiplied by 10,000 in order to give the number of cells in millions per mm^3 of blood.

Calculation :

The smallest square have an area of 0.0025 mm^2 and are 0.1 mm deep, being thus 0.00025 in volume. Since 80 such squares are counted, a volume of 0.00025 x 80 or 0.02 has been covered. In order to give the value per mm^3 the number of cells counted must be multiplied by 50. However, since the dilution is 1 to 200, the multiplication factor is 50 x 200 or 10, 000.

2. ENUMERATION OF RETICULOCYTES

Principle:

The granules in reticulocytes are brought by supravital staining – mixture of appropriate dyes with blood while wet. Such stained cells can be enumerated in a total of at least 1000 cells.

Material:

New methylene blue stain : Dissolve 1.0 g of new methylene blue in 100 ml of citrate-saline (one volume of 30 g/l of sodium citrate to 4 volumes of normal saline). Filter it before use.

Alternately, 1 g of brilliant cresyl blue is dissolved in 100 ml of normal saline to which is added 0.4 g sodium citrate.

Procedure :

To 2-3 drops of the stain add 2-4 times the amount of blood which has been collected in EDTA. Mix and incubate at 37°C for 15-30 min. Resuspend the cells by gently shaking the tube and put a small drop on to a glass slide and make a smear (Refer Differential leucocyte count). Air dry and examine under a microscope.

Calculation :

Let the number of reticulocytes counted in 100 fields = X and the total number of RBC in 10 field = Y then,

$$\text{Reticulocyte } \% = \frac{X}{Y \times 10} \times 100 = \frac{X}{Y} \times 10.$$

3. PACKED CELL VOLUME

Principle :

The packed cell volume (PCV) or haematocrit of blood is determined using heparinised capillary tubes (75 x I mm) and microhaematocrit centrifuge.

Procedure :

Blood from the finger tip or collected in EDTA is allowed to run about 1/2 to 3/4th length of the tube The tube is sealed on the opposite end using sealing wax or plasticine. The tubes are then transferred to the high speed microhaematocrit centifuge, and placed in grooves of the centrifuge head. They are centrifuged for 5 min at 11,000 rpm and read on the reader which gives the direct haematocrit value in volumes per cent.

4. RED CELL INDICES

From the estimated values of haemoglobin (Refer Proteins, amino acids and metabolites), RBC count (millions/mm^3) and PCV (volumes per cent). the following indices can be calculated.

i) Mean corpuscular volume (MCV),

$$\text{MCV in femto liters (fl)} = \frac{\text{PCV}}{\text{RBC count}} \times 10.$$

ii) Mean corpuscular haemoglobin (MCH),

$$\text{MCH in pg} = \frac{\text{Haemoglobin}}{\text{RBC count}} 10.$$

iii) Mean corpusular haemoglobin concentration (MCHC),

$$\text{MCHC \%} = \frac{\text{Haemoglobin}}{\text{PCV}} \times 100.$$

Note :

MCHC is the most accurate measure of red cell indices. In macrocytic anaemias MCV and MCH increase, MCHC may be normal or diminished. In microcytic hypochromic anaemia MCV, MCH and MCHC will be decreased.

5. ERYTHROCYTE SEDIMENTATION RATE

Principle :

Erythrocyte sedimentation rate (ESR) is the rate at which RBCs sediment when anticoagulated blood is allowed to stand undistrubed vertically.

Material :

Westergren ESR tube : It is a glass tube of 300 x 2.5 mm dimensions and graduated in mm from above downwards over the lower 200 mm of the tube.

Procedure :

Blood (1.6 ml) may be collected into 0.4 ml of 3.1% trisodium citrate, alternately, EDTA anticoagulated blood can also be diluted with trisodium citrate (4 : 1). This has to be carried out whithin 2 hr of collection of blood. A delay of 6 hr is permitted if stored at 4°C. Soon after the dilution, draw it into the Westergren tube upto the 200 mm mark and place absolutely vertical and leave undisturbed for 1 hr. Note the height of the plasma column and read to the nearest mm at the end of 1 hr. This will give the ESR in mm.

6. ENUMERATION OF WHITE BLOOD CORPUSCLES

a. Total leucocyte count

Principle :

The enumeration of leucocytes is carried out according to the same principle as that of erythrocytes. Leucocytes being less numerous a dilution of only 1 to 20 is used and the diluent is usually one which destroys the red blood corpuscles.

Materials :

1. WBC pipette : A WBC pipette is similar to a red cell pipette with a white coloured glass bead, but calibrated to give 1 to 20 dilution.
2. Diluent fluid : Mix 2.0 ml glacial acetic acid and 1 drop of gentian violet in 100 ml of water.
3. Neubauer counting chamber : Same as that used for erythrocyte count.

Procedure :

The method of counting is similar to that for erythrocytes except that the count is made in 4 large (1 mm^2) corner squares of Neubauer counting chamber.

Calculation :

The total number of cells in 4 squares is multiplied by a factor of $\frac{50 \times 20 \times 1}{1 \times 0.1 \times 4}$ to give the count per mm^3 of blood.

b. Differential leucocyte count

Materials :

1. Leishman's stain : Dissolve 0.2 g of Leishman dye in 100 ml of acetone free methanol at 50°C for 15 min with occasional shaking. Cool and filter the solution.
2. Buffered water : Make up 50 ml of 0.066 M Sorenson's phosphate buffer, pH 7.0 (Mix 3.89 ml of 9.1 g/l of KH_2PO_4 and 61.1 ml of 9.5 g/l of Na_2HPO_4 or 11.9 g/l of $Na_2HPO_4 . 2H_2O$) to 1 l with water.

Preparation of blood smear : Place a drop of blood collected either directly or in EDTA (within 3hr of collection) on to clean glass slide about 1–2 cm from one end. A spreading slide is placed at an angle of 45° approximately on the slide and move it backwards so that it makes contact with the blood. The drop should spread out quickly along the line of contact of the spreader with the slide. With a single swift forward movement spread the blood along the surface of the slide. The drop of blood taken should be just sufficient to produce a smear of 3–4 cm in length. The ideal thickness is such that there is some overlap of red cells throughout much of the film length.

Procedure :

Place the slide flat on two glass rods over a sink. Cover the slide with the stain and wait for 2–3 min. Dilute the stain by the drop by drop addition of buffered water and stain for a period of 5–7 min. Drain off the stain and wash in water. Air dry and view it under microscope.

Choose the ideal portion of the smear for counting by scanning the smear under low power. Start counting under high power or oil immersion objective from the edge of the smear moving the smear towards the centre. Identify and count the leucocytes as they appear. Shift the slide laterally and then move towards to the edge. Repeat this movement till a total of 100 cells are counted. Express the values of different morphological types as the percentage.

7. OSMOTIC FRAGILITY OF ERYTHROCYTES

Principle :

Blood is added to the hypotonic solutions of varying concentrations of NaCl in the proportion of 1 to 100. The test is carried out at room temperature and the extent of haemolysis is measured colorimetrically.

Material :

Stock solution of buffered NaCl : Dissolve 100 g NaCl, 27.31 g Na_2HPO_4 and 4.86 g NaH_2PO_4. $2H_2O$ in water and make upto 2 l. This solution is osmotically equivalent to 10% NaCl.

In preparing hypotonic solutions for use, it is convenient to make first a 1% solution from the 10% stock solution by dilution with distilled water. Further dilutions giving solutions equivalent to 0.85, 0.75, 0.65, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20 and 0.10% NaCl are made for the test. It is convenient to prepare 50ml of each dilution. The solutions keep well at 4°C for some weeks but should be discarded if moulds develop.

Procedure:

For the test one needs heparinised venous blood or defibrinated blood. Oxalated or citrated blood would not do because of the additional salts added to the blood. To 5 ml volumes of a suitable range of hypotonic solutions is added 0.05 ml volume of blood and mixed well immediately. The tubes are allowed to stand at room temperature (20°C) for at least 30 min, then remixed and centrifuged for 5 min at 2000 rpm. The amount of haemolysis in each tube is compared with that in a 100% lysis tube (0.1% NaCl) using a photoelectric colorimeter provided with a yellow green filter. The supernatant from the 0.65% NaCl tube is used as the blank. The supernatant can usually be poured by decantation from the tubes into the colorimeter cell. With a good colorimeter as little as 1% lysis can be determined. If necessary measured volumes of the supernatants can be diluted with equal volume of 0.10% NaCl.

Blood may be added to the hypotonic solutions by means of a glass capillary automatic pipette calibrated to deliver 0.05 ml. Alternatively, a straight pipette calibrated to contain 0.05 ml may be used, but this method although accurate if a dry pipette is used for each addition, is tedious. A more rapid but less accurate method is to add a large drop of blood to each tube.

Reference :

Practical Haematology. J. V. Dacie and S. M. Lewis (Eds.). Churchill Livingstone, 5th Edn. (1975).

XVI. ANIMAL EXPERIMENTATION

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1. CHOICE OF ANIMAL SPECIES FOR EXPERIMENTATION

a. Factors influencing the selection

In experimental work involving the use of laboratory animals, there are a number of factors that govern the choice of the animal species to be used.

i) The presence or absence of physiological systems and response similar to those in man, for example, the dog does not acetylate aromatic amines, and the monkey and the guinea pig require exogenous ascorbic acid while the rodents do not.

ii) The ease of handling, housing, breeding and maintenance in healthy and normal environment and cost of production, for example, the rabbit is easy to handle and administer intravenous injections.

iii) Experience in terms of past use in other laboratories and by other investigators. To give few examples, the rabbit skin is relatively sensitive to irritants so that a great deal of experimentation on skin irritants have been conducted in rabbit. The monkey is useful in behavioural work. Nerve demylination is best investigated in the chicken. Sensitisation studies in animals are best conducted in the guinea pig.

iv) The ease of experimentation in a short duration. To cite a few instances, for long term studies species having a relatively shorter life span allows one to examine the effects of a chemical or exposure to irradiation etc.,through several generations or an entire life span.

Mouse and rat have been the most standardised and popular of all laboratory animals. Rabbits, guinea pigs and dogs perhaps would be classified next.

b. Factors affecting the experimentation

The use of animals in various investigations demands rigorous investigational discipline. Temperature, humidity, ventillation, nutrition, housing conditions, cleanliness, disease control and personnel care are best a few of the more obvious factors requiring the attention of the investigator.

The recommended housing and environmental conditions for laboratory animals are given in Table-A.

It is necessary that the investigator should be aware of some of the normal breeding and related parameters of laboratory animals. This aids in the proper selection of experimental animals of appropriate sex and age (Refer Table-B).

In addition to this some of the haematological, electrolyte and biochemical reference values of normal laboratory animals are useful in clinical and biological investigations in which experimental animals are used to study human diseases. The range of normal values for laboratory animals can provide criteria for the selection of appropriate experimental animal species for various studies. Some of the reported normal values are presented in tabular form (Refer Tables - C and D).

2. NUTRITIONAL REQUIREMENTS OF ANIMALS AND FORMULATION OF DIETS

a. Nutritional requirements

Adequate nutrition is one of the important environmental factors influencing the ability of laboratory animals to attain their genetic potential for growth, reproduction, longevity and response to stimuli.

Genetic and environmental factors influence the dietary requirement of experimental animals. There are considerable differences in the nutrient requirements of different species (Refer Table - E). Apparent differences in nutrient requirements among different strains of the same species of animals have been demonstrated. The nutrient requirements for most species of laboratory animals change with various stages of the life cycle. Adjustments in nutrient concentrations, the kinds of ingredients and methods of preparations must be considered when formulating diets for laboratory animals

b. Classification of diets

Diets for laboratory animals are classified according to the degree of refinement of the ingredients.

Natural Ingredients:

Diets formulated with appropriately processed whole grains such as wheat, corn or millet, or ingredients that have been subjected to limited amounts of refinement such as fish meal, soya bean meal or wheat bran are referred to as natural ingredient diets (Refer Table - F). Disadvantages of using them for animals involved in research include; the inability to control completely the nutrient concentration, variation in nutrient concentration from batch to batch, difficulty in altering composition to study a particular nutrient and the potential for contamination with pesticide residues, heavy metals or other agents that might alter the response to the experimental treatment.

Purified diets:

Diets formulated with refined ingredients, such as casein (as a source of protein), sugar or starch (as a source of carbohydrate), vegetable oil or lard (as a source of fat) and certain forms of cellulose (as a source of fibre) are designated as purified diets. Chemically pure inorganic salts and vitamins are added to such diets. Planned nutrient concentrations, with minimal variation, can be readily achieved in a purified diet. Advantages of using purified diets are the ability to reproduce nutrient concentrations or to alter them for induction of nutritional deficiencies or excesses. The potential for chemical contamination of these diets is low. Unfortunately, they are not readily consumed by all species.

Chemically defined diets:

Chemically pure compounds such as amino acids, sugars, triglycerides, essential fatty acids, inorganic salts and vitamins are used to prepare these diets. These are useful in studies where strict control of nutrient concentrations is essential, but have been found to be too expensive for general use.

c. Formulation of diets

Ingredients used in laboratory animal diets must be of known nutrient composition, be readily obtainable and palatable to the species involved. More than one ingredient should be used as a source of each class of nutrients when natural ingredient diets are formulated. This will produce diets of higher quality, because it tends to minimise the variations in nutrient composition of a single ingredient and increase palatability. Nutrient losses occur during various feed processing procedures, such as sterilisation or as a result of interactions between nutrients and these should be adequately compensated.

The ingredients have to be ground into fine particles, blended in the amounts specified in the formula, mixed, made into a physical form acceptable to the species involved, and stored in containers until used.

Ingredients used in large amounts are added directly while those used in small quantities such as vitamins and minerals are added through premixes (Refer Table-G).

Separate vitamin and mineral premixes are used to minimise destruction of vitamins by oxidation reaction catalysed by minerals. This will also ensure that specific concentrations of these nutrients are distributed uniformly throughout the diet. Premixes should be prepared with one of the major ingredients as a carrier such that atleast 1 percent of the premix has to be add to the diet.

Diets for laboratory animals can be presented in different physical forms, such as meal or wash (wet or dry) as flaked or baked or as pellets or as semimoist or gel forms.

Note:

Diet should be stored under low temperature and humidity. Areas where diets are stored or processed should be kept clean and enclosed to prevent entry of wild rodents and other pests. Atmost care must be taken to avoid any contamination by any rodenticides, insecticides, hormones, antibiotics or fungiments into the diet.

d. Feeding practices

There are different methods of feeding animals.

Ad libitum feeding : Feed and water are made available for the animal at all times and it can consume as much as it needs.

Pair-feeding : This method of feeding is practiced in nutritional experiments. For a paired feeding trial, the animals should be grouped in pairs, with each member of a pair being as similar as possible in body weight, of the same sex and preferably from the same litter. One animal of each pair is assigned to the experimental group, the other to the control. The number of such pairs in any trial will depend upon a number of factors. Each animal should be assigned a separate cage containing its feed and water. When the trial is actually underway, the feed consumption of both animals in a pair should be weighed each day. Usually, the animal fed the deficient rations will consistently eat much less than the control. The quantity of deficient diet consumed by the test animal is assessed every day. The counter part in the paired fed group will be given equivalent quantity of nutritionally adequate diet.

Restricted feeding : In studies where maximum longevity is vital (cancer research, gerentology etc.) the animals are fed on nutritionally adequate diets, but slightly less than the daily normal ration.

3. ANAESTHESIA

Sedatives, analgesics and general anaesthetic agents are utilised in the humane handling of animals when surgical procedures are undertaken.

For proper induction and maintenance of anaesthesia the following precautions need to be taken.

Handling : The animal should always be handled gently and calmly. Prolonged excitations will disturb circulatory and metabolic state and induce a degree of shock in animals. Attempts to anaesthetise a struggling animal may produce abnormal response.

Fasting : A period of fast sufficient to empty the stomach should be implemented prior to anaesthesia in order to prevent regurgitation and aspiration of gastric contents. A period of 12 hr fasting for most mammals and birds is recommended.

Anticholinergics :

Drugs that block parasympathetic stimulation to cardiopulmonary systems and that reduce salivary secretion are frequently used in combination with other sedatives and analgesics as premedication to general anaesthesia. Preanaesthetic medication facilitates the induction of anaesthesia and reduces risk.

Tranquilisers :

Tranquilisers produce psychological calming without physiological depression. Tranquilisers are useful as they can be used on a wide range of species. Narcotics produce hypnotic and analgesic effects including a significant depression of the cardiovascular and respiratory systems and an alteration in the thermoregulatory mechanism. Canine and primate species are the only ones in which sedation is consistently produced by narcrotics.

General anaesthetics :

Ketamine hydrochloride is most commonly used as an anaesthetic for restraining the animals. When this is used there will be excessive salivation in animals which can be controlled with atropine. Smooth recovery will be facilitated if the animal is left undisturbed in a darkened environment.

Barbiturates are commonly used for the induction and/or maintenance of general anaesthesia. Barbiturates are potent respiratory depressants and their effects on the cardiovascular system are variable. The barbiturates are grouped according to duration of action, into three groups:

(i) Long acting (eg. phenobarbital) (ii) short intermediate acting (eg. pentobarbital) and (iii) ultrashort acting (eg. thiopental, methohexital).

Only the short and ultrashort acting drugs are commoly used in anaesthesia. Anaesthetic duration with short/intermediate barbiturates is approximately 2–4 hr. The effects of ultrashort barbiturates range from 15–30 min. Variation in dose response and duration of effect of barbiturates is extreme within and between species.

Chloralose and urethane are frequently used to produce light anaesthesia. Administration is intravenous and recovery time is very prolonged.

Inhalent anaesthetics

Inhalent anaesthetics give a relatively rapid onset and recovery. Inhalent anaesthetics can be administered by means of a simple nose cone for the performance of short procedures. However, for more extended surgery special anaesthetic equipment are needed.

Inhalent anaesthetics commonly used in experimental animal surgery includes, diethyl ether, halothane, methoxy fluorane and nitrous oxide.

The details on the commonly used anaesthetics, analgesics and narcotics, dosage, route of administration etc. are furnished in the Tables – H and I.

Anaesthetic agents frequently affect the cardiovascular, respiratory and thermoregulatory mechanisms, in addition to the central nervous system. Every effort should be made to maintain the circulation, respiratory blood gases and the body temperature of the anaesthetised subject within the normal physiological limits. Hypothermia may occur during exposure to anaesthetic gases in small animals. This may result in death or delayed recovery from the anaesthetic. Hypothermia may be counteracted to some extent, by placing the animal on a warm water circulator or other devices that help in conserving body heat.

4. TECHNIQUES AND SITES OF BLOOD COLLECTION

In various experimental animals, this varies with the animal species and the quantity of blood required. The common methods of blood collection from laboratory animals are briefly described here.

a. Rats

Tail and toe clippings : Small quantities of blood (0.2 – 0.3 ml) can be collected by clipping the tip of the tail or toe and aspirating directly from the site. The animal has to be under anaesthesia during this method. The animal, including the tail should be kept warm by means of a lamp and gently restrained. The tip of the tail is cleansed with alcohol and when dry, the tip is snipped with a clean scalpel and the blood collected in a pipette or directly on to a slide or tube, discarding the first few drops. The tail must not be massaged or the sample will be diluted with tissue fluids.

Tail vein puncture : The rat is placed on a flat surface under a bright light. The tail is grasped between the thumb and the index finger. The tail is first swabbed with xylol and then with disinfectant (70% alcohol) and then is dried with a sponge. One or 2 ml syringe with 25 - 27 guage and 1/2 – 1 inch needle is used. The veins are visualised laterally and dorsally on the tail. The needle is introduced near the distal portion with the bevel up. This allows one to observe directly as the needle enters the vein and gentle aspiration can be applied. If subcutaneous haemorrhage occurs, it is better to move the site anteriorly along the vein. This is a difficult technique and requires much practice to gain proficiency.

Incision techniques : With the animal under anaesthesia the jugular vein can be entered after a ventrolateral neck incision. The small saphenous vein or the femoropopliteal vein, after incisions over the caudolateral aspect of the rear leg, can be punctured for collection of smaller quantities of blood. The femoral vein may be easily entered by incising the skin of the groin at the junction of the leg and the abdomen.

Cardiac puncture : Large blood samples can be collected by cardiac puncture. This is best performed under anaesthesia, ether or barditurate being acceptable. The anaesthetised animal is restrained in dorsal recumbency by a restraint device. The heart is located under the 5th to 6th ribs by

palpation with the index finger of the left hand, the left thumb being held on the rat's right side. A 2-5 ml syringe with a 19-25 mm (3/4 - 1 inch), 24-26 guage needle is used for bleeding. The needle is inserted at 45° into the thoracic cavity at this point until the heart is felt throbbing against the needle, which is then pushed into the ventricle and the sample withdrawn. The syringe and needle can contain an anticoagulent if necessary. By this method upto 13 ml of blood can be drawn from a rat weighing around 350g.

Bleeding from the orbital sinus : Collection of blood from the orbital sinus is the most efficient method and the one which causes least stress to the animal. This technique is best performed under anaesthesia, however it can be done without its use. A capillary pipette or microhaematocrit tube with a polished tip is passed by the side of the eye into the orbit so as to puncture some of the fragile vessels of the ophthalmic venous plexus. The rat is grasped by the back of the neck and the loose skin of the head is tightened with the thumb and middle finger. The animal is held securely to a flat surface. With the aid of the index finger, the eye is made to bulge slightly by further traction of the skin adjacent to the eye. The tip of the pipette is then inserted in the medial angle (inner corner) of the eye ball and the orbit. The tube is slid alongside the eyeball to the ophthalmic venous plexus. The plexus is reached at a depth of 4-5 mm. The polished tip prevents damage to the eye ball. After puncture of the plexus vessels slight withdrawal of the pipette enables the orbital cavity to fill with blood and the pipette fills by capillary action. Blood can be collected on to a tube held underneath the capillary. Upto 2-3 ml blood can be collected by this method. Bleeding usually stops on withdrawal of the pipette, on reestablishment of the normal ocular pressure on the venous net work. Rat can be bled from both orbits repeatedly for several months without any sign of blindness or other serious damages.

b. Mice

Tail and toe clipping : Small quantity of blood can be collected (0.1 – 0.2 ml) by clipping the tail tip or a toe and aspirating directly from the site (for technique Refer under Rats).

Tail vein puncture : Same as for rats.

Jugular vein puncture : By this method blood samples can be drawn repeatedly for several weeks if necessary. The mouse is held by the skin at the back of the neck and the head is extended by fastening a gauze over its upper incisors and holding this between the fingers of the left hand. The hair on the neck and upper thorax region is removed and both jugular veins are easily seen. A 26-guage needle is used to enter the distended vessel.

Cardiac puncture : Technique is same as for rats. By heart puncture, about 0.7 ml blood can be obtained from a 25 g mouse that has a total blood volume of 2.25 ml.

Bleeding from the orbital sinus : The procedure is exactly similar as that described for the rats.

c. Hamsters

Blood can be drawn from the hamster by venous plexus of the orbit method described for rats. The tail vein of the hamster can be used for repeated blood sampling. The jugular or femoral vein on the side of the hind leg or the heart puncture method can be used to collect large amounts of blood. However, the animal should be anaesthetised before these procedures are performed. Heart puncture yields about 5 ml of blood from a 95 g hamster.

d. Guinea pigs

Small samples of blood (usually less than 0.25 ml) may be obtained by venesection of the marginal ear vein. The veins on the dorsal side of the ear are the most suitable for this purpose. If larger

quantities (about 5 ml) are collected a suction bell can be used. A superficial vein on the medial side of the thigh or the jugular vein may also be used. Repeated small samples can be obtained from the orbital venous plexus.

Larger blood quantities can be collected by cardiac puncture while the animal is under anaesthesia. The method is as follows:

The anaesthetised animal is placed on its back between two plastic bags nearly filled with sand. Hold the sternal region of the guinea pig with the left second finger placed at the anterior end of the sternal bone and the thumb at the xiphoid process. The index finger is then placed exactly in the middle between thumb and second finger in an interval between two left ribs as near the sternal bone as possible. This indicates the insertion point of the needle. The index finger is moved a little to the left and the needle (20 guage, 1½ inch in length) is introduced with the tip perpendicular to the animal. The needle is introduced about 1.5–2.0 cm, the plunger is withdrawn and the blood runs into the syringe. Small corrections in the position of the needle during the bleeding may be necessary. In this way, upto 10 ml of blood may quite easily be taken and only very few animals die of accidental bleeding. Death may happen to an animal suddenly disturbed by noice, which may awaken it from the anaesthesia with the needle in its heart.

The advantage of the method is that it is quick and may be carried out by a single person. The anaesthesia need not be deep as no time is used for restraining with strings and for locating the insertion point estimated by pulsation of the heart.

e. Rabbit

Ear vein puncture: Blood can easily be collected from the marginal ear vein. The area around the vein is shaved and disinfected with 70% alcohol before the vein is nicked with a razor blade. Blood may also be drawn into a syringe. The ear is grasped between the thumb and index finger, with the index finger beneath the ear. Using a 24 – 26 guage needle, with bevel up, the vein is punctured at a site along the vein immediately above the index finger. Very little aspiration must be applied at this site in order to prevent collapsing of the vein over the needle point. Gently rotating the syringe sometimes helps prevent the vein from collapsing. After collection the flow often continues but it can be stemmed by placing a little cotton wool over the vein.

Cardiac puncture : Large quantities of blood may be obtained by cardiac puncture. The animal is anaesthetised and placed in a dorsal position. The left thorax is clipped and swabbed with disinfectant. The thorax is then held between thumb and fingers and the point of strongest beat is palpated. The site of puncture is the third left intercostal space, about 4 mm from the sternum. A needle of 19 – 20 guage, 1¼ inches long, is introduced immediately at this point with gentle aspiration applied to the syringe plunger. About 20 ml of blood can be drawn at a time from an adult rabbit without any harm.

Bleeding from the orbital sinus: Heparinised microhaematocrit tubes (75 mm long by 1.3 or 1.4 mm diameter) are used. One end of the tube is broken off to reduce the length to 50 mm. The rough cutting edge is left as such. The tubes are sterilised.

The rabbit is restrained by an assistant and held on its side with all limbs fully extended. The head is firmly held with one hand and the eyelids are partially retracted with the thumb and forefinger. The tip of the capillary tube is placed in the dorsal junction of the bulbar and palpebral

conjunctivas, midway between the medial and lateral canthi of the eyes. The long axis of the capillary tube is directed somewhat ventrally and caudally in relation to the skull. The fibrous conjunctiva is penetrated by thursting the tube inward while rotating it between the thumb and the forefinger. The tube enters the venous sinus which is at or behind the equitorial region of the eye ball adjacent to the bony orbit. An adequate blood flow into the tube is obtained by seeking optimal effects of gravity and venous pressure by turning the rabbit's head such that the outer end of the capillary tube is lowered. The tube is removed and digital pressure is applied above the eye lid to stop blood flow. If clotting occurs during the procedure, it is repeated in the other eye.

f. Cats

Ear vein puncture: Small samples can be collected from the external ear into a glass capillary tube. The cat should be wrapped in a towel with its head protruding. A small fragment is cut from the edge of the ear, or a vein is nicked with a razor on the dorsal side of the ear. The vessels are clearly outlined after rubbing the ear with ether or xylol. In some cases, it may be necessary to remove the hair by shaving or with a depilatory agent.

Cephalic vein puncture: The cephalic vein is one of the more commonly used sites for blood collection in small quantities. The vein is located by constricting the area on the dorsal aspect of the front limb at the level of the elbow. The area is clipped and shaved (electric clippers are to be avoided since vibrations upset a concious cat and blood values alter in a frightened animal) and swabbed with a disinfectant sponge. A 21 – 24 guage, 1 inch needle is recommended. Samples are more easily and rapidly obtained if the individual constricting the vessel at the elbow will alternately relax and contract his grip on the vessel during the sampling procedure.

Jugular vein puncture: Anaesthesia is not required for this technique. The animal is restrained in dorsal or lateral recumbency with the head extended. The vein is constricted with the thumb at the point of the thorax on either side. Holding the syringe with needle toward the thorax (bevel up) as near as possible in a plane parallel with the vein, the vein is entered with a 20 – 22 guage, $1\frac{1}{2}$ inch needle. This vein has a tendency to roll with the needle and it sometimes is easier to enter if a series of quick thursts are used.

Recurrent tarsal vein puncture: Anaesthesia is not necessary for this technique. The animal must be restrained in either right or left lateral recumbency. The assistant then grasps and constricts the rear limb approximately at the level of the knee joint. The operator clips the hair from the lateral surface of the leg approximately for 10 cm begining at the tarsal joint. The vein is seen to course proximally from the anterior toward the posterior surface of the limb. The vein is more easily entered with a 19 – 20 guage, $1\frac{1}{2}$ inch needle at the point where it begins to turn up to the gastrocnemius muscle.

Femoral vein puncture: Percutaneous puncture of the femoral vein is comparatively easier. The vein lies caudal to the femoral artery in the femoral canal and is easily entered without distension of the vien. It is located by palpating the femoral pulse. The vein is constricted above the point of entry and a 21 – 25 guage 1 inch needle is used. The cat forms hematomas easily, if a vein is once entered and the needle withdrawn accidentally and it is best to attempt puncture at a site proximal to the point of earlier puncture.

Cardiac puncture: This is done under anaesthesia. The animal is restrained in left lateral recumbency. The right thorax is clipped, shaved and a disinfectant solution applied. The fourth

intercostal space is located and the point of strongest heart beat is palpated. Usually, atleast 1½ inch needle is necessary, the guage being dependent on the size of the sample. The needle is inserted through the fourth intercostal space into the heart.

g. Dogs

Blood samples from the dog can be collected by puncturing the cephalic vein, jugular vein, recurrent tarsal vein or femoral vein. The procedures are same as described for the cats. Large quantities can be collected by cardiac puncture. For cephalic vein puncture, a 19-guage, 1½ inch needle is used.

Femoral artery puncture : Percutaneous puncture of the femoral artery is easily accomplished and does not require anaesthesia of the animal. The animal must be restrained in dorsal recumbency with the legs retracted laterally. The area of the junction of the leg and the abdomen is then clipped and cleansed with disinfectant. The femoral pulse is palpated and an 18 - 22 guage needle is introduced immediately beneath the point where the pulse is felt. Large quantities of blood may be obtained at this site and upon completion of the sampling subcutaneous haemorrhage is easily controlled by pressure over the area for approximately 2 - 4 min.

h. Monkeys

Cardiac puncture : This is best accomplished under anaesthesia. The animal is restrained in dorsal recumbency. The left anterior thorax is clipped, shaved and disinfected. The site of the puncture is located at the fourth intercostal space anteriorly alongside the sternum. The point of strongest beat is either palpated or auscultated as the needle is introduced immediately beneath this point. A 18 - 20 guage, 2 inch needle is used.

Femoral vein puncture : Blood is most conveniently withdrawn from monkeys through the femoral vein. The animal is securely restratined, the leg is clipped along its medial aspect between the knee and abdomen. The pulse in the femoral artery is next detected in the femoral triangle. The vein is situated slightly medial to the artery. A 19 guage 3.2 cm needle is introduced into the vein and blood is withdrawn slowly. Occasionally the operator may inadvertently introduce the needle into the artery. When this occurs care must be taken to prevent the development of a hematoma by applying pressure to the area until bleeding ceases.

Small saphaneous vein puncture : Blood can be sampled from the great saphaneous vein which is on the medial side of the lower leg. Holding the leg with the hand below the knee causes congestion and clearly outlines the vein. With a 21-24 guage, 1 inch needle these veins can be entered and very gentle aspiration applied.

i. Chickens

Small quantities of blood can be collected by puncturing the comb or by snipping off a comb tip with scissors. Hemostasis can be effected by searing the wound with a hot metal instrument. Large amounts of blood can be drawn from the wing vein (vena cutanea ulnaris) on the inside of the elbow joint. A few feathers will have to be removed from the site before blood is drawn. Hemostasis is effected by pressure with slight displacement of the skin.

5. EUTHANESIA

Euthanesia means the killing of an animal with a minimum of physical or mental suffering. This is used during the termination of an experiment when the animals are to be disposed off. There are many methods available for this purpose such as physical methods like cervical dislocation, rapid freezing in liquid air or nitrogen, stunning, electrocution, induction of hypoxia and chemical methods like inhalation of ether, carbonmonoxide, carbondioxide or injection of concentrated doses of anaesthetics like pentobarbitone. The choice of a particular methodology is based on whether an experimenter needs the tissues of the animal and for what purposes.

Some recommended methods for small laboratory animals are as follows :

Mice : Mice may be killed humanely by cervical dislocation. The mouse is placed on a flat surface, a pencil or pair of forceps is placed across the back of the neck and dislocation is effected by sharp backwards jerk on the base of the tail.

Alternatively, mice can be killed in chambers or bags with carbondioxide or carbonmonoxide taking care to ensure the pups are dead as well as adults. Pentobarbitone injections (IP 150 mg/Kg) also can be employed.

Rats : Young rats may be stunned on the edge of the bench or be killed by neck disarticulation. Adults are killed in an euthanesia chamber with carbondioxide or carbonmonoxide, or by intraperitoneal injections of pentobarbitone (100 mg/Kg).

Hamsters : These are best killed with carbondioxide or carbonmonoxide in a killing chamber or with pentobarbitone (IP 150 mg/Kg).

Guinea pigs : Dislocation of the neck is the simplest and the most humane method in guinea pigs. The right hand is placed over the front of the animal's head and the neck is gripped between finger and thumb. The arm is swung downward vertically to one side and the weight of the animal dislocates the neck at the bottom of the drop. Alternatively, they can be killed with carbondioxide or carbonmonoxide in a chamber or with pentobarbitone (IP 90 mg/Kg).

Rabbits : These may be killed by a sharp blow at the back of the neck with a short length of wood. Alternatively, the neck may be dislocated if the head is bent sharply backward at the same time as the legs and body are jerked sharply downward with the other hand. Both methods provide instant and painless death if carried out properly.

The most satisfactory method is injection of pentobarbitone (60 mg/Kg) into ear vein after ensuring proper physical restraint. Rabbits struggle if exposed to volatile agents in euthanesia chambers and even appear apprehensive if exposed to carbondioxide or carbonmonoxide.

Cats : Young cats are best killed by striking the base of the skull on a hard surface such as the bench edge or rim of a metal dust bin. This method is quick and least distressing to the animal. Cats of all ages can be killed with carbondioxide or carbonmonoxide in a suitable container and undergo minimal discomfort. Alternatively, pentobarbitone (180 mg/ml) can be given by the intravenous, intraperitoneal or intrathoracic routes at a dose rate of 50 mg/Kg.

Dogs : The simplest and most humane way of killing dogs is by rapid intravenous injection of a concentrated solution (180 mg/ml) of pentobarbitone at 40 mg/Kg. Respiratory paralysis and cardiac arrests are produced within seconds with no untoward vocalisation or struggling.

Primates : After monkeys have been physically restrained they can be given sedative doses of ketamine (20 mg/Kg), before injecting lethal dose of pentobarbitone (50 mg/Kg) by intracardiac route. The handler is thus exposed to minimum hazard, a consideration which may be particularly important if the animals are being destroyed because of disease.

6. COMMON SURGICAL TECHNIQUES

a. Liver biopsy

(i) Rat

Ether anaesthesia is administered by placing the animal in a desiccator containing ether. The rat is then tied to a board by its limbs in supine position. It is usually necessary to give a few whiffs of ether frequently during the operation.

The hair on the adomen are removed from area of about 4 cm below the xiphisternum and the skin is swabbed with alcohol. A clean incision about 1.5 cm long is made on the ventral abdominal wall on the mid line from the xiphisternum passing through the various layers of the abdominal wall including the peritoneum. Left lobe of the liver usually presents itself, after retraction of the muscles on either side. It is gently held between the thumb and finger by a forceps, without crushing the tissue and a slice of about 2 mm thickness and 7 mm in length is cut by scissors. Bleeding is negligible. The liver slice is placed immediately in 20 volumes of 10% neutral buffered formalin or any other suitable fixative.

Abdomen is closed in layers. Peritoneum can be included with the muscle layer and closed by a single continuous suture by a fine linen thread. Skin, however, should be closed separately, preferably by multiple interrupted simple sutures by a fine linen thread, which could be removed after 7 days.

Note :

While sterile conditions are not necessary, absolute cleanliness should be observed.

(ii) Monkey

Fast the animal overnight and the biopsy is performed in the early morning next day.

Pentothol sodium 25 to 30 mg/Kg body weight is injected intravenously but slowly. Intraperitoneal injection is also satisfactory but takes 5–10 min for the animal to get under anaesthesia. Anaesthesia by ether is inconvenient.

The skin over the right mid axillary line overlying the 7th or 8th ribs is shaved and cleaned with soap and water and swabbed with alcohol. The animal is placed in supine position. The Vim-Selveman needle (sterilised by boiling) with the stylet, is taken in the right hand, while the left hand is used to immobilise the liver by pushing it towards right side of the animal. The needle is plunged in the 9th or 10th intercostal space in the mid axillary line for a depth of 1 or 2 cm. After penetrating the muscle, the resistance of the liver tissue is characteristic and can be recognised with a little experience. Then the stylet in the needle is withdrawn and the inner needle (with the two halves of cutting edges) is inserted into the needle and the liver is pierced. One or two complete rotations of the latter are performed to cut out a cylindrical piece of the liver tissue. If resistance is encountered to an abnormal extent, it means that

the needle is not in liver The needle may in that case be withdrawn and one or two more attempts can be made safely. The whole needle is quickly drawn out, the inner needle removed. The two halves are gently separated and the tissue coaxed out into 10% buffered neutral formalin (for histopathology). The animal should be watched for local bleeding and puncture sealed by tincture benzoin. Usually, about 1 – 2 cm length of liver tissue can be obtained.

Note :

Failures are not uncommon, particularly in animals with body wasting.

b. Partial hepatectomy

The animal is anaesthetised with pentothol sodium (3 mg/100 g body weight) injected intraperitoneally and the abdomen opened with a midline incision, extending from the xiphisternum to about 1 inch downwards. The liver becomes immediately visible and depending upon the number of lobes to be removed, they are gently pulled out through the incision. A silk ligature is placed around the lobes at the point where all the lobes join and the ligature tied. The lobe or lobes are then cut distal to the ligature with a pair of sharp scissors. If properly tied, no bleeding occurs from the cut end of the lobes. The abdomen is then closed in layers and the animal returned to the cage. The whole procedure is done with due regard to asepsis. No particular postoperative care is indicated. As much as 70% of the liver may be safely removed.

Note :

If more then one lobe is to be removed, it is preferable to ligate the lobes separately, instead of tying them together in a bunch, though this can also be done. There is, however, a risk of the ligature slipping if the latter is done and in such an eventuality the control of bleeding becomes very difficult.

c. Ovariectomy

The operation is performed under ether anaesthesia taking all aseptic precautions. The sterilised instruments are kept in a petridish containing 90% alcohol. The animal is made to lie on its side and a skin incision is made about 2 cm lateral to the midline on the anterior abdominal wall running approximately from infront of the hip to a point somewhat anterior to the upper pole of kidney. Extending the points of the foreceps through the snip, the ovary which is found embedded in fat lying just below the dorsal muscle mass is drawn out through the incision and a ligature placed around the uterus just below the fallopian tube and tied tightly. The uterus is then cut through on the ovarian side of the ligature and the ovary is removed. The muscle and skin are sutured separately with a fine silk thread and the wound is dressed with tincture benzoin. The skin sutures may be removed on the 7th day and the animal may be taken on the experiment.

d. Orchidectomy

The animal is anaesthetised with pentothol sodium (25 mg/Kg body weight IV). The testicular skin and surrounding areas are cleaned well with soap and water and then an alcohol swab is applied.

A separate scrotal incision for each testis is more convenient than a single median incision. The skin over the testis is stretched and a vertical incision, 1/2 – 3/4 inch is made. The incision is extended into the underlying faciae and down to the layer covering the testis (tunica vaginalis testis). The testis is freed from all around by a careful separation from its covering layers and it is brought out through the

skin incision. The spermatic cord is then cut between two ligatures. After securing all the bleeding points the skin incision in the scrotum is closed. The same procedure can be repeated on the other side, if bilateral orchidectomy is indicated. The whole operation should be performed under proper aseptic conditions.

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TABLE–A

Housing and environment

SPECIES (weight)	SPACE per ANIMAL			TEMPERATURE °C		R.H %	Ventilation changes/ hr	B. T. U. Animal/ hr
	Single floor area	Minimal height	Group or loose housing	Room/Cage	Pen/free ranging			
MONKEY < 7 Kg	0.40 m^2	0.9 m	2-3 m^2	22-25	18-29	45-60	10-15	60-200
(Macaque) > 15 Kg	0.75 m^2	1.2 m	perches					
CAT	0.28 m^2	0.76 m perch	0.56 perches	20-22	15-25	45-60	10-18	25-30
RAT < 150 g	150 cm^2	18 cm	fem+litter	20-25	15-29	50-55	10-20	40
> 150 g	250 cm^2		800 cm^2					
CHICKEN				65-70	12-27	45-70	5-15	30
DOG < 12 Kg	0.75 m^2	0.8 m	1.5 m^2	18-21	5-25	45-55	8-12	80-150
> 15 Kg	1.20 m^2	0.9 m	2.0 m^2					
GERBIL	116 cm^2	15 cm	pair+litter 900 cm^2	15-24	0-30	40-50	8-10	4.0
MOUSE < 20 g	65 cm^2	13 cm	fem+litter	22-25	20-30	50-70	8-12	0.6
> 20 g	100 cm^2	15 cm	160 cm^2					
GUINEA PIG < 350 g	300 cm^2		500 cm^2	16-20	13–31	50-60	4-8	5.6
> 350 g	650 cm^2		800 cm^2					
HAMSTER	100 cm^2	fem+litter 18 cm	900 cm^2	21-24	20-30	45-65	6-10	2.5
RABBIT < 4 Kg	0.37 m^2	0.38 m	fem+litter	16-20	10-28 shade	40-50	10-20	30-40
> 4 Kg	0.46 m^2		0.93 m^2					

B. T. U. - British Thermal Unit

TABLE — B

Parameters of reproduction in common laboratory aminals

	Rat	Mouse	Syrian hamster	Cat	Gerbil	Rabbit	Guinea pig	Dog
Male								
Age at pairing (weeks)	10-11	6	6-8	7-9**	10-12	6-9**	3**	10-12**
Duration ofE.R.A.*(month)	6-9	6-9	12-15	5-7+	12-18	3-6+	2.5-3+	5-7+
Female								
Age at pairing (weeks)	10-11	6	6-8	7-8**	10-12	6-7**	3**	12-14**
Duration ofE.R.A.(months)	6-9	9-12	9-12	5-8+	12-15	1-3+	1.5-2.5+	4-5
Type of estrous cycle	Polyestrous	Polyestrous	Polyestrous	Polyestrous	Polyestrous	Polyestrous	Polyestrous	Monoestrous
Length of estrous cycle (days)	4-5	4-5	4	14-21	4-6	No regular cycle	13-18	21-28
Breeding season	All year	All year	All year, with decrease Oct-Feb.	Jan-Oct.	All year	All year with decrease in summer and winter	All year	Biannual, usually spring and fall
Length of gestation (days)	20-22	19-21	16-17	64	24-26	31	63	63
Litter size at birth (mean)	8	8	7	4	5	7	3	6
(range)	7-14	6-12	6-9	1-8	4-8	6-10	1-6	4-8
Wt. of young at birth (g)	5-6	1-3	2	95-140	3	30-70	85-90	Variable
Weaning age (days)	21	21	21	6-7@	21	6-8@	2-3@	6-8@
Weaning wt (g)	40-50	10-12	35-40	703-800	11-18	800-1500	180-240	Variable
Return of estrous after parturition	Post-partum estrous, regular cycle at end of lactation	Post-partum estrous, regular cycle at end of lactation	Past-partum estrous, regular cycle end of lactation	4th week of lactation	Post-partum estrous	4th week of lactation	Post-partum estrous then regular cycle	Next breeding season

*E. R. A. Economic Reproductive Ability

** = Months

@ = Weeks

+ = Years

TABLE — C

Heamatological and electrolyte values

Mean values and ranges

SPECIES	R B C x 10^6/mm^3	Hb g/100ml	P C V ml%	Blood vol. ml/Kg	W B C x 10^3/mm^3	Sodium mEq/l	Potassium mEq/l	Inorganic phosphorus mg/100ml	Calcium mg/100ml
MONKEY	5								
(M. fascicularis)	4-6	10-12	35-43	55-75	5-10	146-152	4-5	5-5.4	9.5-10
	7.3	10.5	40.5		17.0	151	4.8	6.3	10.7
CAT	5-10	8-15	24-45	45-75	5-20	147-156	4-6	4.5-8.1	5-13
	8.5	14.2	45.9		9.8	147	6.2	7.9	11.5
RAT	6-10	11-17	40-50	50-65	5-13	140-156	5.4-7.0	3-11	5-14
	3.1	10.1	34.3		19.7	155	5.3	7.0	16.8
CHICKEN	2-4	7-13	25-45	60-90	9-31	148-163	4.6-6.5	6.2-7.9	9-24
	6.8	17.0	53.6		12.6	147	4.5	4.2	9.9
DOG	5.5-8.5	12-18	37-59	75-100	6-18	135-180	3.5-6.7	2-9	2.9-11.7
	8.5	15.0	47.0		10.2	—	3.3-6.3	3.7-8.2	3.7-6.2
GERBIL	7-10	10-17	43-52	60-85	7-22				
	6.5	13.5	40.8		8.6	144	6.0	4.9	9.9
RABBIT	5-8	8-17	31-50	45-70	3.0-12.5	138-160	3.7-6.8	2.3-6.9	5.6-12
	5.2	14.3	43.6		11.2	123	5.0	5.3	10.2
GUINEA PIG	3-7	11-17	37-50	65-90	6-17	120-149	3.8-7.9	3-7.6	5.3-12
	7.2	16.4	50.8		8.1	131	5.0	5.7	9.9
HAMSTER	4-10	13-19	39.59	65-80	5-11	106-146	4.0-5.9	3.4-8.2	5-12
	9.2	11.1	41.8		13.6	136	5.3	6.0	6.4
MOUSE	7-13	10-14	33-50	70-80	6-17	128-186	4.9-5.9	2.3-9.2	3.2-8.5

TABLE — D

Clincal biochemistry reference values

Mean values and ranges

SPECIES $\bar{x}$ range	Glucose mg/100m	B. U. N[1]. mg/100ml	Cholesterol mg/100ml	Total Protein g/100ml	Albumin g/100ml	S.G.O.T[2] I.U./l	S.G.P.T.[3] I.U./l	Alkaline phosph. I.U./l
MONKEY (M. fasciculars)	60-90	18-28	100-150	7.5-8.7	2.4-3.4	34-56	21-39	15-35
	117	26	105	6.0	2.8	18	19	12
CAT	60-145	20-30	75-150	4.5-8	2-4	7-29	9-30	3-21
	75	14.5	27	7.6	3.7	63	24	87
RAT	50-135	5-29	10-54	4.7-8.2	2.7-5.1	46-81	18-30	57-128
	164	2.0	96	6	2.7	148	13	35
CHICKEN	125-200	0.5-6	52-148	5-7	2-3.5	88-208	10-37	25-44
	86	16.5	189	6.8	3.4	52	37	17
DOG	64-120	8-22	95-275	5.7-7.8	2-4	33-75	16-67	7-25
	94	21	—	7.9	3.1	—	—	—
GERBIL	40-140	17-31	90-130	5-17	2.5-4.5	—	—	12-37
	132	18.5	26	6.8	3.3	71	65	130
RABBIT	78-155	9-32	20-83	5-8	2.5-4	42-98	49-79	90-170
	92	23.5	30	5.2	2.6	47	42	70
GUINEA PIG	82-107	9-32	16-43	5-6.8	2.1-3.9	27-68	25-59	55-108
	69	22	53	7.1	3.3	100	24	17
HAMSTER	33-118	12-26	10-80	4-8	2.5-4	38-168	12-36	3-31
	89	19.5	64	6.2	3	36	13	19
MOUSE	63-176	14-28	26-82	4-8.6	2.5-4.8	23-48	2-24	10-28

1. Blood Urea Nitrogen.
2. Serum Glutamic Oxaloacetic Transaminase.
3. Serum Glutamic Pyruvic Transaminase.

TABLE—E

Recommended dietary allowances for different animal species

Animal species	Nutrient (percentages)			
	Protein	Carbohydrate	Fat	Fibre
Cat	30-40	NE	25-30	NE
Chicken	15-20	60	1 (EFA)	NE
Dog	20-25	60	10	NE
Guinea pig	18	45-48	4.7	10
Hamster	15	60	5	3
Monkey	15	61.8	5	3-5
Mouse	21.2	60.7	7.6	5.5
Rabbit	16-20	48	5-10	11
Rat	20	60	5	5

NE — Not essential, EFA-Essential fatty acid.

Salt mixture (4%) and vitamin mixture (1%) should be added to these diets to meet the requirements of minerals and vitamins of these species.

TABLE – F

Composition of stock diets

Ingredients	Diet I (%)	Diet II (%)
1. Wheat flour	15	62
2. Roasted bengalgram dhal	58	28
3. Groundnut flour	10	—
4. Skim milk powder	5	—
5. Casein	4	1
6. Refined oil	4	5
7. Salt mixture	4	4
8. Vitamin mixture	0.2	0.2
9. Vitamin C	—	0.05

Diet I is for rats, mice and hamsters.

Diet II is for monkeys, rabbits and guinea pigs.

An extra amount of 20 g of sprouted bengalgram, 15 g groundnuts and one banana a day for monkeys; 20 g sprouted bengalgram and 50 g lucerne grass for rabbits and 25 g of sprouted bengalgram and 25 g of lucerne grass for guinea pigs.

TABLE—G

Composition of mineral mixture (g/100 g of salt mixture)

1.	Calcium carbonate $CaCO_3$	—	38.1400
2.	Cobalt chloride $CoCl_2.6H_2O$	—	0.0023
3.	Cupric sulfate $CuSO_4.5H_2O$	—	0.0477
4.	Ferrous sulfate $FeSO_4.7H_2O$	—	2.7000
5.	Magnesium sulfate $MgSO_4.7H_2O$	—	5.7300
6.	Manganese sulfate $MnSO_4.H_2O$	—	0.4010
7.	Potassium iodide KI	—	0.0790
8.	Potassium phosphate monobasic KH_2PO_4	—	38.9000
9.	Sodium chloride NaCl	—	13.9300
10.	Zinc sulfate $ZnSO_4.7H_2O$	—	0.0548

Dry and grind to a fine powder before weighing. Grind in a mortor a portion of NaCl with KI. Grind together the reminder of NaCl with other salts. Finally, add the NaCl-KI mixture. Store in a cool dry place.

Composition of vitamin mixture

One g of vitamin mixture contains the following :

1.	Vitamin A+	—	2000 IU
2.	Vitamin D+	—	200 IU
3.	Vitamin E	—	10 IU
4.	Vitamin K (Menadione)	—	0.5 mg
5.	Thiamine	—	0.5 mg
6.	Riboflavin	—	0.8 mg
7.	Pyridoxine	—	0.5 mg
8.	Calcium pantothenate	—	4.0 mg
9.	Niacin	—	4.0 mg
10.	Inositol	—	10.0 mg
11.	Para aminobenzoic acid	—	10.0 mg
12.	Biotin	—	40.0 μg
13.	Folic acid	—	0.2 mg
14.	Vitamin B_{12}	—	3.0 μg
15.	Choline chloride++	—	200.0 mg

Mix all the above ingredients and add sufficient amount of starch to make up to 1 g.

+ Vitamin A and D are available as commercial preparation, vanitin. Add appropriate volume of this concentrate to the oil before mixing it with the diet.

++ Prepare a 50% (W/W) mixture of choline chloride with starch and add at 0.2% level at the time of preparation of the diet.

Store in a cool, dark and dry place.

TABLE – H

Tranquiliser and anticholinergic drug dosages

SPEICES	Atravet mg/Kg	Atravet Route	Thorazine mg/Kg	Thorazine Route	Sparine mg/Kg	Sparine Route	Milltown mg/Kg	Milltown Route	Valium mg/Kg	Valium Route	Atropine Sulfate mg/Kg	Atropine Sulfate Route
CAT	0.2-0.5 1-3	IM IV PO	1-2 2-4	IM IV PO	2-4	IM IV	20 50	IP PO	1.0 max5mg		0.02- 0.05	SC IM IV
CHICKEN/PIGEON			0.5-1.5	IM	0.5	IM						
DOG	0.1-0.5	IM IV SC	1-2 2-4	IM IV PO	2-4	IM IV	200- 400	PO	1.0 max20.0	IM IV	0.02- 0.06	SC IM IV
GUINEA PIG			0.5	IM	0.5-1	IM	100	IM	2.5	IM IP	0.02- 0.06	SC IM IV
HAMSTER/ GERBIL			0.5	IM	0.5-1	IM	100	IM	5.0	IP	0.02- 0.05	SC IM IV
MONKEY	0.5-1	SC IM	1-3 2-5	IM PO	2-4	IM	100- 400	PO	1.0	IV IM	0.05	SC IM IV
MOUSE			0.5	IM	0.5	IM	100	IM	5.0	IP	0.02- 0.06	SC IM IV
RABBIT	1.0	SC IM	1-3	IM	1-2	IM	50-100	IM	1.0	IM IV	0.1-0.2	SC IM IV
RAT			0.5	IM	0.5-1	IM	150	IM	2.5	IP	0.02- 0.06	SC IM IV

PO – oral; SC – subcutaneous; IP – intraperitoneal; IV – intravenous; IM-intramuscular.
Atropine should be administered 35–50 min prior to surgery SC or IM

TABEL—I

Analgesic, hypnotic/sedative and injectable anaesthetic agents-dosages

SPECIES	Aspirin	Pethidine	Fantanyl Droperidol	Xylazine	Saffan	Pantobarbital	Thiopental	Ketamine HCl	Chloral Hydrate	Urethane
	mg/Kg (PO)	mg/Kg (IM)	mg/Kg (IM)	mg/Kg (IM)	mg/Kg (IV)	mg/Kg (IV)	mg/Kg (IV)	mg/Kg (IM)	mg/Kg (IV)	mg/Kg (IP)
CAT	10 BID	2-4 (SC) 5-10 (PO)		1-2	9 12 (IM)	25	28	15-30	300	1.25 (IV) 1.50
DOG	10 TID	5-10 (SC) (PO)	0.2-0.5	1-2	contrain-dicated	30	25	—	125	1.0 (IV)
GUINEA PIG	270 (IP)	2	0.5-10		10-20	30 40 (IP)	20 55 (IP)	22-44	200-300 (IP)	1.5
HAMSTER	240 (IP)	2	0.1 (IP) of 1:10 dil			35 (IP)	20 40 (IP)	—	200-300 (IP)	—
MOUSE	400 (SC)	2	0.1 (IP) of 1:10 dil		10-20 90 (IP)	35 50 (IP)	25 50 (IP)	22-44	400 (IP)	—
MONKEY	100	11	0.1-0.2	1-2	6-9 12-18 (IM)	35	25 60 (IP)	15-40	—	—
RABBIT	500	2	0.2-0.3	30 (IV)	6-9	30 40 (IP)	20	22-44	—	1.0 (IV)
RAT	450	2	0.2-0.4 (IP)		10-20	25 50 (IP)	20 40 (IP)	22-44	300 (IP)	.75

Frequency – once daily unless indicated as BID - 2 x daily or TID – 3 x daily.
PO–oral; SC - subcutaneous; IM – intramuscular; IP - intraperitoneal; IV – intravenous.

XVII STATISTICAL METHODS

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1. CONCEPTS IN STATISTICS

Statistics :

Statistics is the science dealing with the designing of experiments and surveys leading to the collection of numerical facts and the method of analysing, interpreting and presenting these numerical facts. Also statistics are measurements which characterise samples.

Biometry or its equivalent biostatistics may be defined as the application of statistics as a science to biology.

Parameter and statistics :

A parameter is a constant describing a population, while statistics is a quantity describing a sample, namely a function of the observations.

In order to distinguish between parameters and statistics, it is customary to denote the parameters with Greek letters and the statistics with Latin letters. Thus, the mean and standard deviation of a population are written as μ and σ, while those of a sample are written as $\bar{x}$ and SD.

Probability :

Probability level (P) is used in evaluating the results. It refers to the probability that a figure as large as or larger than one obtained, would occur by chance alone. It is usually expressed in decimal form, ie , 0.20, means the odds are 20 in 100 or 20 percent. For example, if a statistical test result is significant at the 0.05 probability level ($P = 0.05$), this means that such a value would occur by chance alone less than 5 times in 100 trials.

Significance level :

It is frequently desirable to choose an arbitrary cut off level for probability. The usual cut off levels are, 0.05, often termed significant, 0.01 and 0.001 often termed highly significant. Convenient tables are available for various sized samples at each of these levels.

Degrees of freedom :

Degrees of freedom (df) is defined as the number of ways in which a group of numbers can vary independently. For example, we take 20 measurements of haemoglobin levels. If we calculate the mean ($\bar{x}$) of the 20 values, the series now have (n-1) df, or 19 df, because the twentieth value in the series cannot be changed without altering the mean.

Presentation of data :

Data can be presented in the form of tables, which should be simple and clear. Also graphs may be drawn to give a quick overall idea of the results. The following are the graphs frequently used.

Types of graphs :

1. Bar diagram
2. Pie diagram
3. Pictogram

for qualitative and quantitative data of a discrete type.

4.	Histogram	for quantitative data of a continuous type.
5.	Frequency polygon	
6.	Line diagram	to study trends.
7.	Scatter or correlation diagram	to indicate the relationship between two variables.

2. NORMAL DISTRIBUTION

In a great many statistical problems dealing with biological data, the frequency distribution is supposed to follow what is called the normal curve of error. This curve is expressed in a mathematical form in terms of the population mean and standard deviation (measure of scatter of observations about the mean). The population mean (μ) and standard deviation (σ) are called parameters. Using the data collected from samples drawn from the populations, estimates of the population parameters are calculated. Probability helps to state how far these estimates agree with the population values.

The curve of normal distribution is a symmetrical bell shaped curve representative of many types of numerical distributions. The curve has a standerd shape. It has many uses in statistics. It is called the Gaussian curve or the curve of Demorvre, Bernouilli, Laplace and Gauss.

This is useful for testing the differences between mean values of large samples.

3. MEASURES OF CENTRAL TENDENCY

An average is a typical value which tends to sum up or describe the mass of data. The average is a measure of the location of central tendency. Several types of averages can be defined, the most common being the arithmetic mean or briefly the mean, the median and the mode.

a. Arithmetic mean

The arithmetic mean of a set of 'n' numbers, say $x_1, x_2, x_3 \ldots\ldots\ldots, x_n$ is denoted by $\bar{x}$ (read 'x bar') and is defined as $\bar{x} = \dfrac{x_1 + x_2 + \ldots\ldots\ldots x_n}{n} = \dfrac{\sum_{i=1}^{n} x_i}{n}$

b. Median

The median is the value of the middle number when the numbers are arranged in order of magnitude or size. If there is an even number of values, the mid point is taken as the arithmetic mean of the two central values.

The median is an average of position, while the arithmetic mean is a calculated average. The median is computed from ungrouped data as follows :

(i) Arrange the items according to magnitude.

(ii) Record the size of the middle value. If there are an even number of items, there will be two central values, therefore, the arithmetic mean of these two central values is taken as the median.

Example : For the set of numbers 3, 4, 5, 4, 6, 8, 10, 8, 8, median is computed below.

In order of magnitude, the numbers are 3, 4, 4, 5, 6, 8, 8, 8, 10. The central value is 6 ie. the fifth one from the beginning. The median value is 6.

Example : The set of numbers 5, 5, 7, 9, 12, 15, 11 and 18 has a median 1/2 (9+11) =10. ie. The average of the two central values when arranged in order they are 5, 5, 7, 9, 11, 12, 15, 18. Median is $\frac{9+11}{2} = 10.$

c. Mode

The mode of a group of observations is the value around which the observations tend to be most heavily concentrated. The mode can be calculated using the following relationship existing between the mean, median and mode values of a set of data.

$$\text{Mean—Mode} = 3\ (\text{Mean-Median})$$

Example : The mean and median values of protein for sorghum hybrid samples are 12.2 and 10.8g respectively. The mode value is calculated as follows :

Mode =Mean—3 (Mean-Median) =12.2-3(12.2—10.8)=8.

4. MEASURES OF DISPERSION OR VARIATION

There are various measures of dispersion or variation utilised. Details of some useful measures are described here.

a. Range

This is the simplest possible measure of dispersion and it is the difference between the largest and the smallest value.

b. Standard deviation

The degree to which numerical data tend to spread about an average value is called the variation or dispersion of the data. Of the various measures of dispersion, standard deviation is the one most commonly used.

The standard deviation of a set of 'n' numbers, $x_1, x_2, x_3, \ldots x_n$ with mean value $\bar{x}$ is denoted by 'SD' and is defined as

$$SD = \sqrt{\sum_{i=1}^{n} di^2 \Big/ n-1} = \sqrt{\sum_{i=1}^{n} (xi-\bar{x})^2 \Big/ n-1} = \sqrt{\sum_{i=1}^{n} xi - n\bar{x}^2 \Big/ n-1,}$$

where, i ranges from 1 to n;

$\sum_{i=1}^{n} x_i$ is sum of all values from i = 1 to n and $\bar{x}$ is mean,

$\sum_{i=1}^{n} xi^2$ is sum of squares of all values of x_i, i ranging from 1 to n, di represents the deviations of each of the numbers x_i from the mean $\bar{x}$, $\sum_{i=1}^{n} di^2$ is the sum of squares of deviations of xi from the arithmetic mean $\bar{x}$, i ranging from 1 to n.

Example : The standard deviation of the following data on protein content of local sorghum varieties is 9, 3, 8, 8, 9, 8, 9, 18.

$$\text{Arithmetic mean} = \bar{x} = \frac{9+3+8+8+9+8+9+18}{8} = \frac{72}{8} = 9.$$

$$SD = \frac{(xi - \bar{x})^2}{n-1} = \sqrt{\frac{(9-9)^2+(3-9)^2+(8-9)^2+(8-9)^2+(9-9)^2+(8-9)^2+(9-9)^2+(18-9)^2}{7}}$$

$$= \sqrt{\frac{120}{7}} = \sqrt{17.14} = 4.14.$$

$$\text{or } SD = \sqrt{\frac{\sum xi^2 - n\bar{x}^2}{n-1}} = \sqrt{\frac{(9^2+3^2+8^2+8^2+9^2+8^2+9^2+18^2) - 8 \times 9^2}{7}}$$

$$= \sqrt{\frac{768-648}{7}} = \sqrt{\frac{120}{7}} = \sqrt{17.14} = 4.14.$$

c. Standard error

This is used to compare arithmetic means with one another. The standard deviation of mean values is known as standard error. It is calculated using the following formulae :

$$\text{Standard error (SE)} = \frac{\text{Standard deviation}}{\sqrt{\text{Sample size}}} = \frac{SD}{\sqrt{n}}.$$

The standard error in the previous example is $SE = \frac{4.14}{\sqrt{8}} = \frac{4.14}{2.83} = 1.463.$

d. Coefficient of variation

The standard deviation expressed as a percentage of the mean is known as coefficient of variation.

$$\text{ie. } CV = \frac{SD}{\text{Mean}} \times 100.$$

This is a mere ratio and its magnitude is independent of the units of measurement, used to compare the relative variability of two series of observations.

5. TESTS OF SIGNIFICANCE

There are various types of problems for which the tests of significance are used for drawing conclusions. The types of problems that are common are, comparison of :

(i) sample mean with population mean and (ii) two sample means.

't' Test and 'F' test are commonly used for small samples and normal curve test is used for large samples. The steps involved in general, in the procedure of tests of significance are :

(i) Find the type of problem and the question to be answered.

(ii) Calculation of the SE of mean difference between two sample mean values.

(iii) Calculation of the test criterion. Generally this is given by

$$\text{'t'} = \frac{\text{difference between the mean values}}{\text{SE of difference between the mean values}}.$$

(iv) Compare the value observed in the experiment or study with the predetermined significant level given by the table for various degrees of freedom (df). The number of degrees of freedom is defined as the number of 'n' independent observations in the sample (sample size) minus the number 'k' of population parameters estimated from the sample of observations. In symbols, df = n-k.

a. 't' Test

The most commonly used test in assessing the significance of the difference between two sample means is the 't' test or modified 't' test.

Example -1 ; Comparison of sample mean with the population mean : Ten individuals are chosen at random from a normal population and their weights are found to be 63, 63, 67, 69, 70, 71 and 71 Kg. In the light of these data, discuss the suggestion that the mean weight in the population is 66 Kg.

Solution :

(i) Question to be answered : Is the mean weight of ten individuals chosen at random significantly different from the population mean weight of 66 Kg?

Data given is; size of the sample n = 10, mean of the population $\left(\frac{\wedge}{X}\right)$ = 66 Kg, sample values have the mean $\bar{X}$ = 67.8 Kg, calculated SD = 3.011 and SE = 0.9522.

(ii) Calcuation of test criterion : Test criterion is

$$t = \frac{\left(\frac{\sim}{X} - \frac{\wedge}{x}\right)}{SE} = \frac{67.8 - 66.0}{0.9522} = 1.89,$$

Calculated 't' follows 't' distribution with df of 9.

(iii) Level of significance : For 9 df, at 5% level of significance, $t_{0.05}$ value from the table is 2.62. Calculated value of 't' is less than $t_{0.05}$ = 2.62. Since the calculated value of 't' is less than the theoretical value of 't' at 5% level, the sample mean weight and the proposed mean weight of 66 Kg are more or less same.

(iv) Inference : The data does not provide any evidence of significance against the hypothesis.

Example-2 ; Testing difference between two sample means : Given two random samples with means $\bar{x}_1$ and $\bar{x}_2$ with SDs of SD_1 and SD_2 and sample sizes n_1 and n_2 respectively, test the hypothesis that the population means are more or less same.

To carry out the test, calculate the test criterion,

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{\left(\frac{1}{n_1} + \frac{1}{n_2}\right) SD^2}}$$ where $\bar{x}_1$ and $\bar{x}_2$ are the mean of first and second sample respectively.

$$SD^2 = \frac{1}{(n_1 + n_2 - 2)} [(n_1 - 1) SD_1^2 + (n_2 - 1) SD_2^2].$$

Here 't' follows the 't' distribution with $(n_1 + n_2 - 2)$ degrees of freedom. If the calculated value of 't' is greater than $t_{0.05}$ or $t_{0.01}$, the difference between the sample means is said to be significant at 5% or 1% level of significance; otherwise the data is consistent with the hypothesis that both means are more or less same. 't' Table values for various df are given in the Table-A.

Example-3 : The intelligence quotients (I. Q) of 16 children from one area of a city showed a mean of 107 with a standard deviation of 10, while the I. Q's of 14 students from another area of the city showed a mean of 122 with a SD of 8. Is there any significant difference between the I. Q.'s of the two groups?

Question to be answered : Is there any difference between the mean values of I. Q.'s of two groups of children?

Given data	Group I	Group II
Mean I. Q' s	$\bar{x}_1 = 107$	$\bar{x}_2 = 122$
SD	$SD_1 = 10$	$SD_2 = 8$
Sample size	$n_1 = 16$	$n_2 = 14$

$$SE = \sqrt{\left(\frac{1}{16} + \frac{1}{14}\right) \left(\frac{(1-61)\,10^2 + (14-1)\,8^2}{16+14-2}\right)} = 4.0485.$$

Test criterion :

$$t = \frac{(107-122)}{4.0485} = 3.704.$$

Degrees of freedom $= n_1 + n_2 - 2 = 28$.

Level of significance for 28 df at $t_{0.05} = 2.048$, $t_{0.01} = 2.763$ and $t_{0.001} = 3.674$.

Since the calculated 't' value is greater than theoretical value even at 0.1% level, the difference in I.Q's between two groups of children studied are significant.

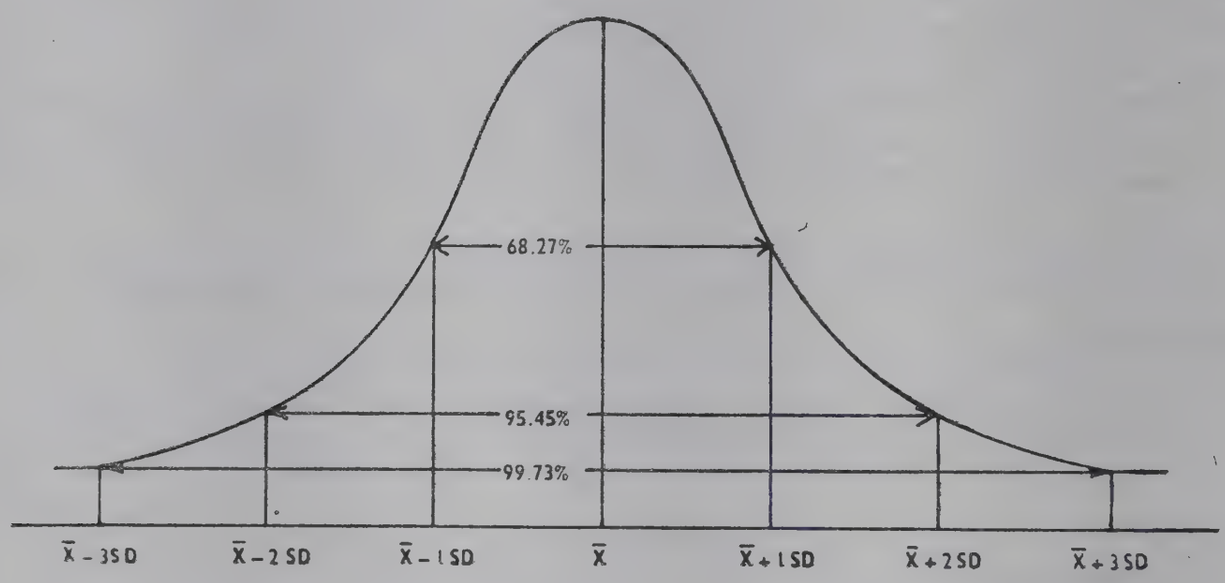
Inference : The study shows that the I.Q's of two groups of children are entirely different from each other.

b. Normal curve test

The mathematical characteristics of many biological values such as heights and weights of men, haemoglobin levels etc., often approximate to what is called a 'normal distribution'. When the heights of a large sample of men are plotted, we get a distribution or a curve. The properties of normal curve are:

(i) it is symmetrical (ii) it is a bell shaped curve (iii) mean = median = mode (iv) mean $\pm$ 1 SD includes 68.27% of the values, mean $\pm$ 2 SD includes 95.45% of the values and mean $\pm$ 3 SD includes 99.73% of the values. These are illustrated in the figure.

NORMAL CURVE WITH LIMITS OF VARIATION



By taking a specific significant level of 5, 1 or 0.1%, we can use normal curve properties to test differences between two large sample mean values. Details of procedure are given below :

Large sample test or normal curve test for comparsion of the means of two samples : Here, under the assumption that large sample has the characteristics of a normal probability distribution, the test is carried out. The details of two samples are provided as below :

Sample	Size	Mean	SD	SE
A	n_1	$\bar{X}_1$	SD_1	$SD_1/\sqrt{n_1}$
B	n_2	$\bar{X}_2$	SD_2	$SD_2/\sqrt{n_2}$

To test difference between $\bar{X}_1$ and $\bar{X}_2$ we have to find out SE of the difference $(X_1 - X_2)$

$$= \sqrt{SE_1^2 + SE_2^2} \qquad = \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}.$$

$$\text{Test criterion} = Z = \frac{(\bar{X}_1 - \bar{X}_2)}{\text{SE of the difference } (\bar{X}_1 - \bar{X}_2)}$$

This 'Z' value is compared with the expected value at 5, 1 or 0.1% levels of significance. Expected values are : $Z_{0.05} = 1.96$ at 5% level, $Z_{0.01} = 2.58$ at 1% level and $Z_{0.001} = 3.20$ at 0.1% level of significance.

If Z is greater than $Z_{0.05}$ or $Z_{0.01}$ or $Z_{0.001}$, we say there is significant difference at 5, 1 or 0.1% level, respectively.

Example : The mean and SE values for height of American and Indian adults are given below. Test whether there is any significant difference between the two racial groups.

Here $\bar{X}_1 = 174.2$, $\bar{X}_2 = 168.3$; $SE_1 = 0.2024$, $SE_2 = 0.1758$; $n_1 = 1164$, $n_2 = 1456$.

SE of the difference, $(\bar{X}_1 - \bar{X}_2) = \sqrt{SE_1^2 + SE_2^2} = 0.2681$.

Test criterion :

$$Z = \frac{\bar{X}_1 - \bar{X}_2}{\text{SE of difference}} = \frac{5.9}{0.2681} = 22.01 \text{ which is greater than } Z_{0.001}.$$

The value of the test criterion 'Z' is very much greater than 3.20 which is the expected value at 0.1% level. Therefore it is concluded that the difference in height between American and Indian adults is highly significant.

c. 'F'-test for the equality of two variances

In the application of students 't' test for the equality of two means, one of the assumptions made is that the population variance of the samples taken are equal. This assumption needs verification before applying the students 't' test. 'F'-test is applied to assess the validity of equality of variances between samples. Details of 'F'-test are provided below :

Samples	Means	Variances	Sample size
A	$\bar{X}_A$	SD_A^2	n_A
B	$\bar{X}_B$	SD_B^2	n_B

Samples A and B are assumed to be independent.

Null Hypothesis : Variances are equal, $SD_A^2 = SD_B^2$

Alternative hypothesis : Variances are not equal.

Test criterion : 'F'-test.

$$F = \frac{\text{larger variance}}{\text{smaller variance}} = \frac{SD_A^2}{SD_B^2} \text{ with df of } n_A - 1;\ n_B - 1.$$

Note :

Take larger variance in the numerator for calculation of 'F'.

Levels of significance : 'F' values at 5, 1 or 0.1% levels of significance are given in Fisher and Yates tables (Refer Tables-B. C and D) for various degrees of freedom.

Conclusion :

If $F > F_{0.05}$, then $P < 0.05$; $F > F_{0.01}$, then $P < 0.01$; $F > F_{0.001}$, then $P < 0.001$ and $F < F_{0.05}$, then $P > 0.05$; where $F_{0.001}$, $F_{0.01}$, and $F_{0.05}$ are theoretical values of 'F' at 5, 1 and 0.1% levels for $n_A - 1$, $n_B - 1$ degrees of freedom. Theoretical values are given in tables.

Example : Haemoglobin levels of normals and PEM children were examined and results are given below :

		n	Mean	SD	SD^2
Normals	:	20	12.4	1.405	1.974
PEM	:	13	10.5	2.851	8.128

Null hypothesis : Variances are equal.

Alternative hypothesis : Variances are not equal.

Test to be applied : 'F'-test.

$$\text{Test criteria : } F = \frac{\text{larger variance}}{\text{smaller variance}} = \frac{8.128}{1.974} = 4.1175.$$

F has 12 and 19 df and for this $F_{0.05} = 2.31$, $F_{0.01} = 3.30$ and $F_{0.001} = 4.97$.

Therefore $0.001 < F < 0.01$, so $0.001 < P < 0.01$.

Variances are significantly different at 0.01 probability level.

d. Modified 't' - test

The ordinary method of using tests of significance for the difference between the means of two independent samples, assumes the two variances of the samples studied are same. We may come across samples violating this assumption of equality of variances. The following procedure may be used if the variances of two samples are significantly different.

$$t^1 = \frac{\bar{x}_A - \bar{x}_B}{\sqrt{SE_A^2 + SE_B^2}}$$ where $\bar{x}_A$ and $\bar{x}_B$ are two sample means and SE_A and SE_B are the standard errors.

Case I : Sample sizes are not equal, ie. n_A not equal to n_B.

If sample sizes are not equal then calculate modified 't^1' as given above.

To find the significance level, we have to find out the $t_{0.05}$ (or $t_{0.01}$) values from tables for the degrees of freedom $n_A - 1$ and $n_B - 1$. Let these values be t_1 and t_2 for $n_A - 1$ and $n_B - 1$ df, respectively.

The significance level of t^1 at 0.05 probability is

$$t^1_{0.05} = \frac{t_1 SE_A^2 + t_2 SE_B^2}{SE_A^2 + SE_B^2}.$$

If $t^1 > t^1_{0.05}$, then the difference between mean values are significant, ie. $P < 0.05$. In a similar way, $t^1_{0.01}$, and $t^1_{0.001}$ can be calculated and level of significance may be found out.

Example : Take,

$$\bar{X}_A = 12.4, \quad \bar{X}_B = 10.5$$

$$SD_A = 1.405, SD_B = 2.851$$

$$n_A = 20, \quad n_B = 13$$

$F = 4.1175 > F_{0.05}$ with 12, 19 df.

Variances are significantly different.

Student 't' is not feasible for application. Modified 't' test is feasible for application. As per modified 't' test the criteria is :

$$t^1 = \frac{\bar{X}_A - \bar{X}_B}{\sqrt{SE_A^2 + SE_B^2}} = \frac{1.9}{\sqrt{0.72395}} = 2.2331.$$

Significance level at 5% is

$$t^1_{0.05} = \frac{t_1 SE_A^2 + t_2 SE_B^2}{SE_A^2 + SE_B^2}$$

where, $t_1 = t_{0.05}$ for 19 df $= 2.093$, $t_2 = t_{0.05}$ for 12 df $= 2.179$

$$t^1_{0.05} = \frac{(2.093)\ (0.09870) + (2.179)\ (0.62525)}{(0.09870 + 0.62525)} = 2.1673; \quad t^1 > t^1_{0.05}$$

Hence the difference between mean values is significant at 5% level ie. $P < 0.05$.

Casell : If sample sizes are same, ie. $n_A = n_B = n_1$, then the procedure for modified 't' test is as given below :

$$t^1 = \frac{\bar{X}_A - \bar{X}_B}{\sqrt{SE_A^2 + SE_B^2}}$$

$t^1_{0.05} = t_{0.05}$ for n-1 df, since $n_A = n_B$

ie $t^1_{0.05} = \frac{t_{0.05} SE_A^2 + t_{0.05} SE_B^2}{SE_A^2 + SE_B^2} = t_{0.05}$, where $t_{0.05}$ is for n-1 df.

If $t^1 > t_{0.05}$, then $P < 0.05$ and if $t^1 < t_{0.05}$, then $P > 0.05$.

Example : Test the significance of difference between mean heights of preschool children studied at two centres. Data are given below :

Centre	No	Mean	SD	SE
A	16	85.4	4.24	1.06
B	16	84.0	2.71	0.61

$F_{0.05}$ for 15,15 df = 2.41.

$F < F_{0.05}$ hence students 't' test is not feasible for application. Modified 't' test is applied. The test criterion value is,

$$t_{0.05} = \frac{\bar{X}_A - \bar{X}_B}{\sqrt{SE_A^2 - SE_B^2}} = 1.11.$$

$$df = \frac{(16 - 1) + (16 - 1)}{2} = 15,$$

$t_{0.05}$ for 15 df = 2.947, $t^1 < t^1_{0.05} = t_{0.05}$ for 15 df.

Hence the differences are not significant ie. $P > 0.05$.

6. ANALYSIS OF VARIANCE

The analysis of variance is a powerful statistical tool for tests of significance. t-Test is used to test the difference between two sample means. In a situation when more than two sample means are to be compared, analysis of variance is used. Analysis of variance (ANOVA) is the "separation of variance ascribable to one group of causes from the variance ascribable to other group".

The assumptions of analysis of variance test are :

(i) observations are independent (ii) parent population from which observations are taken is normal (iii) various treatment and environmental effects are additive in nature and (iv) error variances are homogeneous.

There are analysis of variance procedures developed for one way, two way and three way classification of data on experiments. The application of these procedures depend on the design of experiments utilised for the investigation.

Analysis of variance for one way classification :

Example : Liver weights, expressed as a percentage of body weight for rats fed on four different diets are given below. Are the mean weights in different diet regimens significantly different from each other ? (Completely randomised block design was adopted for the experiment).

	DIETS					
	I	II	III	IV		
	3.4	3.2	3.3	3.6		
	4.0	3.6	3.7	3.9		
	3.9	3.4	3.8	3.8		
	4.0	3.5	3.7	4.2		
	3.6	3.4	3.6	4.2		
	3.8	3.4	4.5	3.9		
	3.8	3.4	3.6	4.0		
n_i	7	7	7	7	28	n
T_i	26.5	23.9	25.2	27.6	103	T
S^2_i	100.61	81.61	90.88	109.10	382.28	S^2
$\bar{x}_i$	3.79	3.41	3.60	3.94	3.69	$\bar{x}$
SD_i	0.2193	0.1215	0.1633	0.2149		

where, n = total of all the animals ; T = grand total of weights of all 28 rats ; S^2 = total sum of squares of 4 samples ; $\bar{x}$ = over all mean of 28 observations.

1. Total corrected sum of squares (T.C.S.S.) $= S^2 - \frac{T^2}{n}$

$$= 382.28 - \frac{(103)^2}{28} = 3.3871$$

2. Sum of squares between diets $= \frac{T_1^2}{n_1} + \frac{T_2^2}{n_2} + \frac{T_3^2}{n_3} + \frac{T_4^2}{n_4} - \frac{T^2}{n}$

$$= \frac{(26.5)^2}{7} + \frac{(23.9)^2}{7} + \frac{(25.2)^2}{7} + \frac{(27.6)^2}{7} - \frac{(103)^2}{28} = 2.5728$$

3. Within sum of squares = T.C.S.S. – Sum of squares between diets = (1)—(2)
$= 3.3871 - 2.5728 = 0.8143$

Degrees of freedom : (a) for diets = No. of diets – 1 = 3 and (b) within sum of squares = n – 1 – df of diets = n – 1 – 3 = 24.

ANOVA TABLE

Source	df	S.S.	M.S.S.	F-ratio
Between diets	3	2.5728	$\frac{2.5728}{3} = 0.8576$	$\frac{0.8576}{0.0339} = 25.30$
Within (error)	24	0.8143	$\frac{0.8143}{24} = 0.0339$	
Total	27	3.3871		

$F_{0.001}$ for 3,24 df is 7.55; $F > F_{0.001}$ hence $P < 0.001$. The mean liver weights in different regimens are significantly different ($P < 0.001$) from each other. It is pertinent to find out which of the two mean values are different from each other. There are many procedures available. The studentised range test procedure is provided in the following paragraphs.

Critical difference (C.D.) at 0.001 = tabulated value 't' for error degree of freedom x $\sqrt{\text{mean error sum of squares}\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}$

$= t_{0.001,24}\sqrt{\frac{2 \times \text{mean error S. S.}}{n}}$ if $n_1 = n_2 = n$

$= 3.745\sqrt{\frac{2 \times 0.0339}{7}} = 0.3687$

If the difference between any two means is greater than the calculated C.D. at 0.1% level is 0.3687 then the two means are significantly different from each other,

Difference between mean values of diets	Result
I - II = 3.79 - 3.41 = 0.38	$P < 0.001$
I - III = 3.79 - 3.60 = 0.19	N. S.
I - IV = 3.79 - 3.94 = 0.15	N. S.
II - III = 3.41 - 3.60 = 0 19	N. S.
II - IV = 3.41 - 3.94 = 0.53	$P < 0.001$
III - IV = 3.60 - 3.94 = 0.34	N. S.

Result:

The mean liver weight in 2nd diet regimen is significantly lower than that of I and IV. In the above manner, whether the mean values are different or not must be examined at 5% and 1% levels also.

For two way or three way analysis of variance and for F – test any standard statistical book may be referred.

7. Chi-square TEST OF ASSOCIATION

The Chi-square test is a simple and direct test of significance. It is suitable for most cases in which the observations can be classified into discrete categories and treated as frequencies. The test can be utilised in testing certain types of associations between two variables.

Examples are :

(i) Association between smoking and cancer (ii) Association between night blindness and infection (iii) Association between nutritional status and infection and (iv) Association between night blindness and conjenctival xerosis.

To test the association between night blindness and xerosis of conjenctiva, the data available on an examination of 50 preschool children are provided in the table below :

		Night blindness		
		Present	Absent	Total
Xerosis	Present	11 (a)	9 (c)	20 (a+c)
	Absent	5 (b)	25 (d)	30 (b+d)
	Total	16 (a+b)	34 (c+d)	50 = N = (a+b+c+d)

$$\text{Chi - square} = \frac{N\,(ad - bc)^2}{(a+b)\,(c+d)\,(b+d)\,(a+c)} = \frac{50\,(11 \times 25 - 5 \times 9)^2}{(20)\,(30)\,(16)\,(34)}$$

$$= 81.035 \text{ (significant)}$$

Chi - square has df equivalent to (No. of columns –1) x (No. of rows - 1) = (2 - 1) (2 - 1) = 1

Tabulated value of Chi - square for 1 df at 5% level is 3.84 which is less than 81.035 ie. Chi - square $= 81.035 > \text{Chi - square}_{0.05} = 3.84$.

So there is association between the two nutritional deficiency signs ($P < 0.05$).

Note :

Two way table has df for Chi - square to be one. For various df theoretical values of Chi - square are available in the table (Refer Table – E).

8. CORRELATION COEFFICIENT

It is often desirable to observe and measure the relation between two or more variables. It may, for instance, be desirable to know whether there is a relationship between changes in ths cost of living and changes in wages; serum protein and calorie intake; the grades of an examination and the intelligence quotient of a group of students; the linear stature and body weights of a group of children and many other similar correlated series of variables.

The association or magnitude of relationship between two variables can be measured by means of correlation coefficient, calculated using the following formulae :

$$\text{Correlation coefficient} = r = \frac{\sum_{i=1}^{n} xi\ yi - \frac{\sum_{i=1}^{n} xi \sum_{i=1}^{n} yi}{n}}{SD_X\ SD_Y\ (n-1)}.$$

where,

X — denotes the values taken by one variable;

Y — denotes the values taken by another variable;

SD_X — is the standard deviation of sample values of 'X' variable;

SD_Y — is the standard deviation of sample values of 'Y' variable;

n — is the sample size;

$\sum_{i=1}^{n} x_i$ — is the total of n sample values of variable 'X'

$\sum_{i=1}^{n} y_i$ — is the total of n sample values of vairable 'Y'

The coefficient of correlation varies between — 1 to + I. The signs + and — are used for positive correlation and negative correlation respectively. The correlation coefficient is a dimensionless quantity ie. it does not depend on the units employed.

In testing the significance of correlation coefficient, calculated values may be compared with the theoretical values at various degrees of freedom (Refer Table F). Each correlation coefficient has a degree of freedom '(n-2)' if the sample size studied is 'n'. If r is greater than $r_{0.05}$ or $r_{0.01}$ at 'n-2' df, it can be taken as significant at respective level of significance.

Example : The table below shows the respective heights 'x' and 'y' of a sample of 12 fathers and their eldest sons. Find the coefficient of correlation between heights of fathers and their eldest sons?

Height (inches)

	Father	Son			
n	x	y	x^2	xy	y^2
1.	65	68	4225	4420	4624
2.	63	66	3969	4158	4356
3.	67	68	4489	4556	4624
4.	64	65	4096	4160	4225
5.	68	69	4624	4692	4761
6.	62	66	3844	4092	4356
7.	70	68	4900	4760	4624
8.	66	65	4356	4290	4225
9.	68	71	4224	4828	5041
10.	67	67	4489	4489	4489
11.	69	68	4761	4692	4624
12.	71	70	5041	4970	4900

$\sum_{i=1}^{12} x_i = 800$ $\sum_{i=1}^{12} y_i = 811$ $\sum_{i=1}^{12} x_i^2 = 53418$ $\sum_{i=1}^{12} x_i y_i = 54107$ $\sum_{i=1}^{12} y_i^2 = 54849$

$\bar{X} = 66.67$ $\bar{Y} = 67.58$ $n = 12$

$$SD\ x = \sqrt{\frac{\sum x_i^2 - n\bar{X}^2}{n-1}} = 2.77, \text{ where } i = 1 \text{ to } 12.$$

$$SD\ y = \sqrt{\frac{\sum y_i^2 - n\bar{Y}^2}{n-1}} = 1.88, \text{ where } i = 1 \text{ to } 12.$$

$$\frac{1}{n-1}\left(\sum x_i y_i - \frac{\sum x_i \sum y_i}{n}\right) = \frac{1}{11}\left(54107 - \frac{800 \times 811}{12}\right) = 3.66$$

$$r = \frac{3.66}{2.77 \times 1.88} = 0.7028$$

The coefficient of correlation between heights of fathers and their eldest sons is + 0.7028.

df $= n - 2 = 10$; $r_{0.05} = 0.576$; $r_{0.01} = 0.708$

Hence, $r > r_{0.05}$; $r < r_{0.01}$ correlation between heights is significant at 5% level.
ie. $0.01 < P < 0.05$.

9. DETAILS OF EXPERIMENTAL DESIGNS

Whether one is experimenting with animals, or human beings and analysis of the results to be carried out, the design of experiment is of central importance. Logically and chronologically, the design of an experiment precedes its analysis, and to a large extent the design determines the validity of the analysis.

In quantitative observations on some scale of measurement, variation is often present and it is not wholly controllable by the investigator. In the physical sciences, such variation may arise mainly from measurement errors or from failure to control the environment. In the biological sciences, variations from organism to another or in the physiological state of a single organism, are likely to be important. One of the central tasks of statistical science is to provide methods of handling data of this type. Design of experiments provides a valuable source of help in handling the valuable material and to arrive at reasonable conclusions.

The statistical aspects of the design (or planning) of an experiment are : (a) selecting the treatments (factors or levels of factors) whose effects are to be studied; (b) specifying a layout for the experimental limits (animals or humanbeings) to which the treatments are to be applied; (c) providing rules according to which the treatements are to be distributed among the experimental limits and (d) specifying what measurements are to be made for each experimental unit. All these things must be accomplished in such a way that the techniques to be used in the analysis of the results are clear, prior to the conduct of the experiment.

An experimental unit might be a plot of land, humanbeing, batch of seeds or species of animals. Experimental units are the smallest divisions of experimental material which are such that any two experimental units may receive different treatment.

In any experimental design there are three basic principles to be carefully observed : (i) randomisation (ii) replication and (iii) reduction of random variation

(i) Randomisation :

If two or more treatments of groups of experimental units are to be compared, one would like the groups to be as similar as possible in the various factors that might influence the level of response. Some of these factors can be recognised in advance. One could then try to match individuals in the various groups; for example, in an animal experiment, by selecting groups of animals of about the same weight and age and, in a clinical comparison, by selecting groups of patients in whom the various degrees of severity of their illness are similarly distributed. But there remains a basic problem; how can one ensure that the groups do not differ in some unmatched characteristic that may well affect the results of the experiment? Allotment of the individuals at random to groups using the random numbers, provides a valuable solution. Random number tables may be made use for this (Refer Table—G).

In an animal experiment, the investigator may carefully select two groups of animals at random and then place the cages containing animals due for treatment 'A' at one end of the room and those for treatment 'B' at the other end.

In clinical comparisons the well known devices of single and double blind administrations of therapies are designed to overcome this problem. Although of course, they are not always both feasible and desirable.

(ii) Replication :

A second basic principle is that of replication, or the use of more then one experimental unit, with a given treatment. Greater the number of units to which each treatment is applied, the more precise the comparison of treatment effects. Sample size is most important to control the magnitude of heterogeneity of a variable (example – variance of mean). A further reason for replication is to extend the range of applicability of the results. Sample size or number of replicates can be fixed with some data on degree of random variation, period of study etc.

(iii) Reduction of random variation :

The third basic principle is the use of techniques of exprimental design for the reduction of random variation or what Fisher often called the " LOCAL CONTROL OF VARIABILITY ". There are so many experimental designs developed for use. Prominent among them are :

(a) Completely randomised design : A very simple form of experimental design in which the treatments are allocated to the experimental units (rats, monkeys or humans) purely on a chance or random basis.

(b) Randomised block design : This is one experimental design in which each block contains a complete replication of the treatments, which are allocated to the various units within the blocks in a random manner and hence allow unbiased estimates of error to be constructed.

(c) Factorial experiment : A complete factorial experiment is an expriment in which all levels of each factor (variable) are investigated in combination with all levels of every other factor. It is customary to denote complete factorial experiments by indicating the number of levels for each factor, thus, a 3 x 4 (or r x c) factorial experiment is a two factor experiment where one factor has three levels (r) and the other has four levels (c). Similarly a 2^3 factorial is a three factor experiment, where each factor has two levels.

(d) Latin square design : This is the generalisation of a randomised block design.

(e) Other designs : There are many designs developed, some more prominent are split-unit designs, sequential designs, cross-over designs etc.

10. SAMPLE SIZE

Basic data required for determination of sample size :

(i) Certain amount of safeguard is required when declaring that there is significant difference between the effects of two treatments. It is the choosing of type I error which is the probability of rejecting a null hypothesis, when it is infact, true. Type II error is the probability of accepting a hypothesis when an alternative is true.

(ii) If one treatment is really superior to another, the amount of difference that would be considered as important to detect is to be made available.

(iii) If there is any real difference in the effects of two treatments, we need the amount of assurance for the experiment to produce a statistically significant difference in the outcome.

(iv) The knowledge about the amount of real difference between the effects of the two treatments is necessary. This can be known through previous experiences.

In the first place, if we need more safeguard about conclusions on data, we need a larger sample. That is, if the type I error is small, we need a larger sample. In other words, the size of the sample would be larger when the safeguard is 99% than when it is only 95%. If only a small difference is expected and if we need to detect that small difference, then we need a large sample.

If we demand a higher assurance of detecting that difference, then the sample size should also be larger. On the other hand, if we are satisfied with a 50 : 50 chance of detecting that difference, than we are generously running a risk and our experiment on many occasions would lead to non - significant results, which are inconclusive and therefore a waste.

Determination of sample size needed for estimating means :

In planning an experiment, designed to estimate a mean, the question of sample size is of major importance. The following method is useful for determining 'n' when a prior estimate of the population standard deviation, is available.

The prior estimate of SD, even a good guess, will be necessary.

If $E = (\bar{x} - \mu)$ is the maximum error to be tolerated with a degree of confidence of $(1 - \alpha)$, when using $\bar{x}$ as an estimate of μ, the required sample size is given by the formula.

$$n = \frac{SD^2 \, (t\, \alpha/2)^2}{E^2}$$

Where, $t\alpha/2$ must be obtained from table values of 't'.

Since 't', depends on 'n' a direct solution of this equation is impossible; however, a simple iterative solution is illustrated in the example.

Example : It is desired to obtain one more estimate of the heights of preschool children, so that one can assert with a probability of 0.95 that the error of this new estimate is not more then 1.5 cm. Based on the data available on the previous study, standard deviation was 3.37 cm. What is the sample size needed?

Given that SD = 3.37; $E = (\bar{x} - \mu) = 1.5$, Substituting in the formula for 'n', it yields that

$$n = \frac{SD^2 \, t^2_{0.025}}{E^2} = \frac{(3.37)^2 \, t^2_{0.025}}{(1.5)^2}.$$

Since this equation cannot be solved directly, one can proceed as follows :

(i) Try $t_{0.025} = 1.96$, the values of $Z_{0.025}$

$$n_1 = \frac{(11.33) \quad (1.96)^2}{(1.5)^2} = 19.8 \text{ or } 20.$$

From the table of 't', $t_{0.025}$ for 19df

(ii) Try $t_{0.025} = 2.093$, $n_2 = \frac{(11.33) \, (2.093)^2}{2.25} = 22.06$ or 23.

From the table of 't'; $t_{0.025} = 2.074$ for 22 df

(iii) Try $t_{0.025} = 2.074$, $n_3 = \frac{(11.33) \, (2.074)^2}{2.25} = 21.7$ or 22.

From the table of 't', $t_{0.025} = 2.080$ for 21 df

(iv) Try $t_{0.025} = 2.080$, $n_4 = \frac{(11.33) \, (2.080)^2}{2.25} = 21.8$ or 22.

Since $n_3 = n_4$; further iteration is unnecessary and the required sample size $n = 22$.

Determination of sample size needed for estimation of proportions :

Let us take another example dealing with percentage or proportion and estimate the size of the sample. Selection of the proper sample size in estimating proportions depends on the maximum error to be tolerated and the degree of confidence with which it is desired that this maximum error not to be

xceeded. The following method is useful for determining 'n' when no prior information about 'P' the roportion to be estimated is available.

If a random sample of size 'n' contains 'x' successes and 'n – x' failures, the statistic

$$Z = \frac{\frac{x}{n} - P}{\sqrt{\frac{P(1-P)}{n}}} = \frac{p - P}{\sqrt{PQ/n}}$$

s distributed approximately as a normal variate with mean zero and variance unity i.e. N (0.1).

If $E = \frac{x}{n} - P$ is the maximum error to be tolerated with a degree of confidence $(1 - \alpha)$, when using

x/n as an estimate of 'p' the require sample size is at most $n = \frac{Z^2\alpha/2}{4E^2}$ where, $Z\alpha/_2$ is to be obtained from the table or normal curve areas.

Example : $Z_{0.025} = Z_{0.05}/_2 = 1.96$ $Z_{0.005} = Z_{0.01}/_2 = 2.575$

Example : Suppose it is necessary to know how many kwashiorkor cases would be required to be at least 95 percent confident that the error in estimating the true proportion of kwashiorkor children treated successfully by means of the sample proportion will not exceed 0.15. The calculation yields.

$$n = \frac{(1.96)^2}{4(0.15)^2} = 42.7 \text{ or } 43$$

Thus if the estimate of P is based on a random sample of 43 observations, one can assert with a probability of 0.95 that the error will not exceed 0.15 regardless of the true value of 'P'.

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TABLE — A

Distribution of 't' - Probability

n	0.1	0.05	0.02	0.01	0.001
1.	6.314	12.706	31.821	63.657	636.619
2.	2.920	4.303	6.965	9.925	31.698
3.	2.353	3.182	4.541	5.841	12.924
4.	2.132	2.776	3.747	4.604	8.610
5.	2.015	2.571	3.365	4.032	6.869
6.	1.943	2.447	3.143	3.707	5.959
7.	1.895	2.365	2.998	3.499	5.408
8.	1.860	2.306	2.896	3.335	5.041
9.	1.830	2.262	2.821	3.250	4.780
10.	1.812	2.228	2.764	3.169	4.587
11.	1.796	2.201	2.718	3.106	4.437
12.	1.782	2.179	2.681	3.055	4.318
13.	1.771	2.160	2.650	3.012	4.221
14.	1.761	2.145	2.624	2.977	4.140
15.	1.753	2.131	2.602	2.947	4.073
16.	1.746	2.102	2.583	2.921	4.015
17.	1.740	2.110	2.567	2.898	3.965
18.	1.734	2.101	2.552	2.878	3.922
19.	1.729	2.093	2.539	2.861	3.883
20.	1.725	2.086	2.528	2.845	3.850
21.	1.721	2.080	2.518	2.831	3.819
22.	1.717	2.074	2.508	2.819	3.792
23.	1.714	2.069	2.500	2.807	3.767
24.	1.711	2.064	2.429	2.797	3.745
25.	1.708	2.060	2.485	2.787	3.725
26.	1.706	2.056	2.479	2.779	3.707
27.	1.703	2.052	2.473	2 771	3.690
28.	1.701	2.048	2.467	2 763	3.674
29.	1.699	2.045	2.462	2.756	3.659
30.	1.697	2.042	2.457	2.750	3.646
40.	1.684	2.021	2.423	2.704	3.551
60.	1.671	2.000	2.390	2.660	3.460
120.	1.658	1.980	2.358	2.617	3.373
∞.	1.645	1.960	2.326	2.576	3.291

TABLE – B
Fisher and Yates Variance Ratio — 5 per cent

n_2 \ n_1	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	161	200	216	225	230	234	237	239	241	242	244	246	248	249	250	251	252	253	254
2	18.5	19.0	19.2	19.2	19.3	19.3	19.4	19.4	19.4	19.4	19.4	19.4	19.4	19.5	19.5	19.5	19.5	19.5	19.5
3	10.1	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39	3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.91	2.84	2.77	2.74	2.70	2.66	2.62	2.58	2.54
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90	2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49	2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.06	2.01	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34	2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32	2.27	2.20	2.13	2.05	2.00	1.96	1.91	1.86	1.81	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30	2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28	2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69
27	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25	2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24	2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22	2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39
120	3.92	3.07	2.68	2.45	2.29	2.18	2.09	2.02	1.96	1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88	1.83	1.75	1.67	1.57	1.52	1.46	1.39	1.32	1.22	1.00

Lower 5 per cent points are found by interchange of n_1 and n_2, **ie.** n_1 must correspond with the greater mean square.

TABLE — C

Fisher and Yates Variance Ratio — 1 per cent

n_2 \ n_1	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	4052	5000	5403	5625	5764	5859	5928	5982	6022	6056	6106	6157	6209	6235	6261	6287	6313	6339	6366
2	98.5	99.0	99.2	99.2	99.3	99.3	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.5	99.5	99.5	99.5	99.5	99.5
3	34.1	30.8	29.5	28.7	28.2	27.9	27.7	27.5	27.3	27.2	27.1	26.9	26.7	26.6	26.5	26.4	26.3	26.2	26.1
4	21.2	18.0	16.7	16.0	15.5	15.2	15.0	14.8	14.7	14.5	14.4	14.2	14.0	13.9	13.8	13.7	13.7	13.6	13.5
5	16.3	13.3	12.1	11.4	11.0	10.7	10.5	10.3	10.2	10.1	9.89	9.72	9.55	9.47	9.38	9.29	9.20	9.11	9.02
6	13.7	10.9	9.78	9.15	8.75	8.47	8.26	8.10	7.98	7.87	7.72	7.56	7.40	7.31	7.23	7.14	7.06	6.97	6.88
7	12.2	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.72	6.62	6.47	6.31	6.16	6.07	5.99	5.91	5.82	5.74	5.65
8	11.3	8.65	7.59	7.01	6.63	6.37	6.18	6.03	5.91	5.81	5.67	5.52	5.36	5.28	5.20	5.12	5.03	4.95	4.86
9	10.6	8.02	6.99	6.42	6.06	5.80	5.61	5.47	5.35	5.26	5.11	4.96	4.81	4.73	4.65	4.57	4.48	4.40	4.31
10	10.0	7.56	6.55	5.99	5.64	5.39	5.20	5.06	4.94	4.85	4.71	4.56	4.41	4.33	4.25	4.17	4.08	4.00	3.91
11	9.65	7.21	6.22	5.67	5.32	5.07	4.89	4.74	4.63	4.54	4.40	4.25	4.10	4.02	3.94	3.86	3.78	3.69	3.60
12	9.33	6.93	5.95	5.41	5.06	4.82	4.64	4.50	4.39	4.30	4.16	4.01	3.86	3.78	3.70	3.62	3.54	3.45	3.36
13	9.07	6.70	5.74	5.21	4.86	4.62	4.44	4.30	4.19	4.10	3.96	3.82	3.66	3.59	3.51	3.43	3.34	3.25	3.17
14	8.86	6.51	5.56	5.04	4.70	4.46	4.28	4.14	4.03	3.94	3.80	3.66	3.51	3.43	3.35	3.27	3.18	3.09	3.00
15	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.89	3.80	3.67	3.52	3.37	3.29	3.21	3.13	3.05	2.96	2.87
16	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.78	3.69	3.55	3.41	3.26	3.18	3.10	3.02	2.93	2.84	2.75
17	8.40	6.11	5.18	4.67	4.34	4.10	3.93	3.79	3.68	3.59	3.46	3.31	3.16	3.08	3.00	2.92	2.83	2.75	2.65
18	8.29	6.01	5.09	4.58	4.25	4.01	3.84	3.71	3.60	3.51	3.37	3.23	3.08	3.00	2.92	2.84	2.75	2.66	2.57
19	8.18	5.93	5.01	4.50	4.17	3.94	3.77	3.63	3.52	3.43	3.30	3.15	3.00	2.92	2.84	2.76	2.67	2.58	2.49
20	8 10	5 85	4 94	4 43	4 10	3.87	3.70	3.56	3.46	3.37	3.23	3.09	2.94	2.86	2.78	2.69	2.61	2.52	2.42
21	8.02	5.78	4.87	4.37	4.04	3.81	3.64	3.51	3.40	3.31	3.17	3.03	2.88	2.80	2.72	2.64	2.55	2.46	2.36
22	7.95	5.72	4.82	4.31	3.99	3.76	3.59	3.45	3.35	3.26	3.12	2.98	2.83	2.75	2.67	2.58	2.50	2.40	2.31
23	7.88	5.66	4.76	4.26	3.94	3.71	3.54	3.41	3.30	3.21	3.07	2.93	2.78	2.70	2.62	2.54	2.45	2.35	2.26
24	7.82	5.61	4.72	4.22	3.90	3.67	3.50	3.36	3.26	3.17	3.03	2.89	2.74	2.66	2.58	2.49	2.40	2.31	2.21
25	7.77	5.57	4.68	4.18	3.86	3.63	3.46	3.32	3.22	3.13	2.99	2.85	2.70	2.62	2.54	2.45	2.36	2.27	2.17
26	7.72	5.53	4.64	4.14	3.82	3.59	3.42	3.29	3.18	3.09	2.96	2.82	2.66	2.58	2.50	2.42	2.33	2.23	2.13
27	7.68	5.49	4.60	4.11	3.78	3.56	3.39	3.26	3.15	3.06	2.93	2.78	2.63	2.55	2.47	2.38	2.29	2.20	2.10
28	7.64	5.45	4.57	4.07	3.75	3.53	3.36	3.23	3.12	3.03	2.90	2.75	2.60	2.52	2.44	2.35	2.26	2.17	2.06
29	7.60	5.42	4.54	4.04	3.73	3.50	3.33	3.20	3.09	3.00	2.87	2.73	2.57	2.49	2.41	2.33	2.23	2.14	2.03
30	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	3.07	2.98	2.84	2.70	2.55	2.47	2.39	2.30	2.21	2.11	2.01
40	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.89	2.80	2.66	2.52	2.37	2.29	2.20	2.11	2.02	1.92	1.80
60	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.72	2.63	2.50	2.35	2.20	2.12	2.03	1.94	1.84	1.73	1.60
120	6.85	4.79	3.95	3.48	3.17	2.96	2.79	2.66	2.56	2.47	2.34	2.19	2.03	1.95	1.86	1.76	1.66	1.53	1.38
∞	6.63	4.61	3.78	3.32	3.02	2.80	2.64	2.51	2.41	2.32	2.18	2.04	1:88	1.79	1.70	1.59	1.47	1.32	1.00

Lower 1 per cent points are found by interchange of n_1 and n_2 ie. n_1 must correspond with the greater mean square.

TABLE — D

Fisher and Yates Variance Ratio — 0.1 per cent

n_2 \ n_1	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	4053*	5000*	5404*	5625*	5764*	5859*	5929*	5981*	6023*	6056*	6107*	6157*	6209*	6235*	6261*	6287*	6313*	6340*	6366*
2	998.5	999.0	999.2	999.2	999.3	999.3	999.4	999.4	999.4	999.4	999.4	999.4	999.4	999.5	999.5	999.5	999.5	999.5	999.5
3	167.0	148.5	141.1	137.1	134.6	132.8	131.6	130.6	129.9	129.2	128.3	127.4	126.4	125.9	125.4	125.0	124.5	124.0	123.5
4	74.14	61.25	56.18	53.44	51.71	50.53	49.66	49.00	48.47	48.05	47.41	46.76	46.10	45.77	45.43	45.09	44.75	44.40	44.05
5	47.18	37.12	33.20	31.09	29.75	28.84	28.16	27.64	27.24	26.92	26.42	25.91	25.39	25.14	24.87	24.60	24.33	24.06	23.79
6	35.51	27.00	23.70	21.92	20.81	20.03	19.46	19.03	18.69	18.41	17.99	17.56	17.12	16.89	16.67	16.44	16.21	15.99	15.75
7	29.25	21.69	18.77	17.19	16.21	15.52	15.02	14.63	14.33	14.08	13.71	13.32	12.93	12.73	12.53	12.33	12.12	11.91	11.70
8	25.42	18.49	15.83	14.39	13.49	12.86	12.40	12.04	11.77	11.54	11.19	10.84	10.48	10.30	10.11	9.92	9.73	9.53	9.33
9	22.86	16.39	13.90	12.56	11.71	11.13	10.70	10.37	10.11	9.89	9.57	9.24	8.90	8.72	8.55	8.37	8.19	8.00	7.81
10	21.04	14.91	12.55	11.28	10.48	9.92	9.52	9.20	8.96	8.75	8.45	8.13	7.80	7.64	7.47	7.30	7.12	6.94	6.76
11	19.69	13.81	11.56	10.35	9.58	9.05	8.66	8.35	8.12	7.92	7.63	7.32	7.01	6.85	6.68	6.52	6.35	6.17	6.00
12	18.64	12.97	10.80	9.63	8.89	8.38	8.00	7.71	7.48	7.29	7.00	6.71	6.40	6 25	6.09	5.93	5.76	5.59	5.42
13	17.81	12.31	10.21	9.07	8.35	7.86	7.49	7.21	6.98	6.80	6.52	6.23	5.93	5.78	5.63	5.47	5.30	5.14	4.97
14	17.14	11.78	9.73	8.62	7.92	7.43	7.08	6.80	6.58	6.40	6.13	5.85	5.56	5.41	5.25	5.10	4.94	4.77	4.60
15	16.59	11.34	9.34	8.25	7.57	7.09	6.74	6.47	6.26	6.08	5.81	5.54	5.25	5.10	4.95	4.80	4.64	4.47	4.31
16	16.12	10.97	9.00	7.94	7.27	6.81	6.46	6.19	5.98	5.81	5.55	5.27	4.99	4.85	4.70	4.54	4.39	4.23	4.06
17	15.72	10.66	8.73	7.68	7.02	6.56	6.22	5.96	5.75	5.58	5.32	5.05	4.78	4.63	4.48	4.33	4.18	4.02	3.85
18	15.38	10.39	8.49	7.46	6.81	6.35	6.02	5.76	5.56	5.39	5.13	4.87	4.59	4.45	4.30	4.15	4.00	3.84	3.67
19	15.08	10.16	8.28	7.26	6.62	6.18	5.85	5.59	5.39	5.22	4.97	4.70	4.43	4.29	4.14	3.99	3.84	3.68	3.51
20	14.82	9.95	8.10	7.10	6.46	6.02	5.69	5.44	5.24	5.08	4.82	4.56	4.29	4.15	4.00	3.86	3.70	3.54	3.38
21	14.59	9.77	7.94	6.95	6.32	5.88	5.56	5.31	5.11	4.95	4.70	4.44	4.17	4.03	3.88	3.74	3.58	3.42	3.26
22	14.38	9.61	7.80	6.81	6.19	5.76	5.44	5.19	4.99	4.83	4.58	4.33	4.06	3.92	3.78	3.63	3.48	3.32	3.15
23	14.19	9.47	7.67	6.69	6.08	5.65	5.33	5.09	4.89	4.73	4.48	4.23	3.96	3.82	3.68	3.53	3.38	3.22	3.05
24	14.03	9.34	7.55	6.59	5.98	5.55	5.23	4.99	4.80	4.64	4.39	4.14	3.87	3.74	3.59	3.45	3.29	3.14	2.97
25	13.88	9.22	7.45	6.49	5.88	5.46	5.15	4.91	4.71	4.56	4.31	4.06	3.79	3.66	3.52	3.37	3.22	3.06	2.89
26	13.74	9.12	7.36	6.41	5.80	5.38	5.07	4.83	4.64	4.48	4.24	3.99	3.72	3.59	3.44	3.30	3.15	2.99	2.82
27	13.61	9.02	7.27	6.33	5.73	5.31	5.00	4.76	4.57	4.41	4.17	3.92	3.66	3.52	3.38	3.23	3.08	2.92	2.75
28	13.50	8.93	7.19	6.25	5.66	5.24	4.93	4.69	4.50	4.35	4.11	3.86	3.60	3.46	3.32	3.18	3.02	2.86	2.69
29	13.39	8.85	7.12	6.19	5.59	5.18	4.87	4.64	4.45	4.29	4.05	3.80	3.54	3.41	3.27	3.12	2.97	2.81	2.64
30	13.29	8.77	7.05	6.12	5.53	5.12	4.82	4.58	4.39	4.24	4.00	3.75	3.49	3.36	3.22	3.07	2.92	2.76	2.59
40	12.61	8.25	6.60	5.70	5.13	4.73	4.44	4.21	4.02	3.87	3.64	3.40	3.15	3.01	2.87	2.73	2.57	2.41	2.23
60	11.97	7.76	6.17	5.31	4.76	4.37	4.09	3.87	3.69	3.54	3.31	3.08	2.83	2.69	2.55	2.41	2.25	2.08	1.89
120	11.38	7.32	5.79	4.95	4.42	4.04	3.77	3.55	3.38	3.24	3.02	2.78	2.53	2.40	2.26	2.11	1.95	1.76	1.54
∞	10.83	6.91	5.42	4.62	4.10	3.74	3.47	3.27	3.10	2.96	2.74	2.51	2.27	2.13	1.99	1.84	1.66	1.45	1.00

* Multiply by 100 to read the value.

Lower 0.1 per cent points are found by interchange of n_1 and n_2 ie. n_1 must correspond with the greter mean square.

TABLE — E
Distribution of Probability — Chi - square

n	.05	.02	.01	.001
1	3.841	5.412	6.635	10.827
2	5.991	7.824	9.210	13.815
3	7.815	9.837	11.345	16.266
4	9.488	11.668	13.277	18.467
5	11.070	13.388	15.086	20.515
6	12.592	15.033	16.812	22.457
7	14.067	16.622	18.475	24.322
8	15.507	18.168	20.090	26.125
9	16.919	19.679	21.666	27.877
10	18.307	21.161	23.209	29.588
11	19.675	22.618	24.725	31.264
12	21.026	24.054	26.217	32.909
13	22.362	25.472	27.688	34.528
14	23.685	26.873	29.141	36.123
15	24.996	28.259	30.578	37.697
16	26.296	29.633	32.000	39.252
17	27.587	30.995	33.409	40.790
18	28.869	32.346	34.805	42.312
19	30.144	33.687	36.191	43.820
20	31.410	35.020	37.566	45.315
21	32.671	36.343	38.932	46.797
22	33.924	37.659	40.289	48.268
23	35.172	38.968	41.638	49.728
24	36.415	40.270	42.980	51.179
25	37.652	41.566	44.314	52.620
26	38.885	42.856	45.642	54.052
27	40.113	44.140	46.963	55.476
28	41.337	45.419	48.278	56.893
29	42.557	46.693	49.588	58.302
30	43.773	47.962	50.892	59.703
32	46.194	50.487	53.486	62.487
34	48.602	52.995	56.061	65.247
36	50.999	55.489	58.619	67.985
38	53.384	57.969	61.162	70.703
40	55.759	60.436	63.691	73.402
42	58.124	62.892	66.206	76.084
44	60.481	65.337	68.710	78.750
46	62.830	67.771	71.201	81.400
48	65.171	70.197	73.683	84.037
50	67.505	72.613	76.154	86.661
52	69.832	75.021	78.616	89.272
54	72.153	77.422	81.069	91.872
56	74.468	79.815	83.513	94.461
58	76.778	82.201	85.950	97.039
60	79.082	84.580	88.379	99.607
62	81.381	86.953	90.802	102.166
64	83.675	89.320	93.217	104.716
66	86.965	91.681	95.626	107.258
68	88.250	94.037	98.028	109.791
70	90.531	96.388	100.425	112.317

TABLE—F

The Correlation Coefficient

Values of the correlation coefficient for different levels of significance

n	0.05	0.02	0.01	0.001
1.	.99392	.999507	.999877	.9999908
2.	.95000	.98000	.999000	.99900
3.	.8783	.93433	.95873	.99116
4.	.8114	.8822	.91720	.97506
5.	.7545	.8329	.8745	.95074
6.	.7067	.7887	.8343	.92493
7.	.6664	.7493	.7977	.8962
8.	.6319	.7155	.7646	.8721
9.	.6021	.6851	.7348	.8471
10.	.5760	.6581	.7079	.8233
11.	.5529	.6339	.6835	.8010
12.	.5324	.6120	.6614	.7800
13.	.5139	.5923	.6411	.7608
14.	.4973	.5742	.6226	.7420
15.	.4821	.5577	.6055	.7246
16.	.4683	.5425	.5987	.7084
17.	.4555	.5285	.5751	.6932
18	.4438	.5155	.5614	.6787
19.	.4329	.5034	.5487	.6652
20.	.4227	.4921	.5368	.6524
25.	.3809	.4451	.4868	.5974
30.	.3494	.4093	.4487	.5541
35.	.3246	.3810	.4182	.5189
40.	.3044	.3578	.3932	.4896
45.	.2875	.3384	.3721	.4648
50.	.2732	.3218	.3541	.4433
60.	.2500	.2948	.3248	.4078
70.	.2319	.2737	.3017	.3799
80.	.2172	.2565	.2830	.3568
90.	.2050	.2422	.2673	.3375
100.	.1946	.2301	.2540	.3211

TABLE — G

Random Numbers

92 63 07 82 40	19 26 79 54 57	53 49 72 38 94	37 70 87 98 62	76 47 16 64 18
79 44 57 87 35	71 54 94 48 43	26 10 46 73 18	00 70 13 49 86	66 75 19 97 23
59 65 47 19 66	27 38 65 00 04	05 35 89 40 31	29 28 11 85 44	52 42 90 60 80
31 52 44 95 87	76 61 23 97 89	13 53 74 80 86	59 48 36 06 80	84 67 55 30 14
06 34 87 69 38	90 37 95 13 92	55 88 77 10 15	09 20 97 91 57	24 44 03 02 44
28 70 35 17 09	94 45 64 83 96	73 78 00 64 36	86 64 16 92 39	57 66 23 01 81
68 10 88 92 66	94 73 09 57 61	75 02 34 84 64	65 54 49 65 83	18 91 11 63 91
99 93 89 07 04	93 62 16 63 30	33 39 39 52 61	95 34 95 17 69	91 61 63 32 38
91 54 37 31 96	34 44 96 35 13	83 83 49 94 11	58 82 64 04 56	69 26 84 85 62
42 10 30 27 81	73 92 05 62 97	72 67 81 22 49	25 27 03 66 78	21 31 37 57 67
17 13 82 75 84	25 29 62 83 87	51 35 06 16 64	37 89 30 53 63	44 14 34 26 77
28 29 71 42 80	54 52 42 16 18	95 32 03 81 74	60 57 90 80 89	94 99 97 84 60
09 33 15 67 12	51 33 30 62 89	75 34 50 88 11	82 71 15 73 92	25 25 23 03 33
31 29 50 42 04	93 71 25 12 87	05 75 47 93 96	87 39 95 17 73	33 07 59 70 61
36 14 61 55 60	27 59 24 20 89	99 28 15 96 40	15 22 19 60 79	09 96 10 53 71
29 55 31 84 32	13 63 00 55 29	02 79 18 10 17	49 02 77 90 31	50 91 20 93 99
23 50 12 26 42	63 08 10 81 91	89 42 06 78 00	55 13 75 47 07	32 94 56 45 22
57 88 88 58 46	67 96 70 78 35	11 31 40 15 45	48 53 51 71 42	08 55 26 74 57
55 33 67 12 64	88 47 20 43 34	63 91 93 63 94	11 19 69 24 70	70 54 32 97 76
10 08 71 00 72	55 98 06 46 88	68 23 92 04 61	89 38 19 38 09	00 79 69 59 45
34 10 18 12 77	66 09 08 88 72	37 81 87 21 42	67 14 05 07 85	21 38 01 67 03
53 36 24 49 40	60 43 02 28 34	14 13 09 65 93	23 29 85 62 03	92 67 11 59 25
82 97 56 61 58	84 73 11 94 36	55 79 06 92 29	28 66 11 36 75	99 31 87 68 73
54 82 78 46 73	22 89 80 80 94	14 32 68 70 38	42 89 22 11 27	30 71 24 84 89
25 46 45 90 98	27 43 68 94 21	80 75 48 99 24	19 09 76 77 37	80 36 80 87 95
67 60 96 02 14	41 47 58 49 50	40 13 30 25 46	09 57 04 56 82	50 16 51 56 09
44 92 17 09 24	61 29 55 11 37	47 59 68 67 35	35 56 17 66 49	18 21 76 34 46
33 17 03 09 72	98 13 09 58 28	49 78 61 33 01	36 08 18 07 61	33 98 56 86 21
84 68 78 54 45	06 20 81 76 54	51 33 30 28 78	35 01 06 75 78	61 57 42 07 49
71 88 65 64 50	36 56 70 93 95	96 95 13 55 07	17 55 53 52 12	69 10 60 16 79
00 47 50 22 24	74 72 21 47 21	40 21 52 13 51	08 59 64 56 25	83 98 16 37 48
25 99 33 88 81	68 36 15 95 60	41 27 46 97 42	40 70 33 79 93	03 43 51 88 73
92 88 25 31 78	99 19 59 38 03	56 98 55 30 89	15 30 55 05 22	55 90 04 30 26
25 13 82 68 10	07 09 31 56 77	60 68 80 44 10	24 50 53 78 90	67 18 66 28 29
84 63 17 18 82	12 99 18 30 28	82 48 49 03 39	91 95 07 45 79	03 53 99 01 22
34 00 39 23 26	12 79 36 14 53	48 12 17 42 71	28 36 36 65 61	75 22 03 59 08
53 77 54 57 49	05 73 48 61 69	42 76 27 01 75	97 31 07 38 94	88 60 61 99 94
59 31 69 78 85	72 80 75 49 66	60 85 91 19 32	35 26 57 16 01	55 57 76 77 15
20 47 96 65 57	50 70 52 69 99	09 85 45 25 91	14 06 33 02 14	19 89 01 92 92
86 18 08 49 31	25 45 52 60 44	02 22 75 20 15	21 82 05 05 99	51 67 16 54 11
21 45 16 80 01	72 71 04 02 61	61 28 11 31 72	63 81 94 89 70	51 73 25 41 13
98 06 26 83 75	80 08 92 41 35	72 35 59 54 28	11 80 82 97 40	81 64 48 66 10
01 78 86 44 29	14 43 09 45 75	75 15 39 45 76	61 39 39 61 92	03 22 73 22 58
62 46 50 48 41	43 27 26 87 02	01 27 40 54 37	22 95 31 89 46	99 05 34 16 90
94 32 43 10 89	84 15 99 29 33	99 98 98 95 00	91 58 60 28 02	69 47 16 82 74
05 79 74 39 84	21 57 50 99 08	70 22 11 97 91	51 57 83 64 32	33 49 47 98 88
10 39 51 42 89	52 18 50 97 21	25 78 93 85 62	54 79 40 48 97	59 01 28 92 51
35 17 75 69 86	25 54 95 97 30	64 71 85 26 30	31 10 06 23 84	49 48 31 14 09
25 63 38 96 19	75 88 29 82 56	02 12 67 20 99	57 18 35 58 87	09 32 07 34 63
16 46 44 82 80	94 25 44 57 77	45 15 06 88 65	11 38 21 17 82	22 69 54 19 88

12 36 72 38 91	31 50 69 07 21	18 71 08 91 40	58 59 59 94 25	22 84 00 82 67
38 89 03 02 39	34 23 72 25 78	74 42 02 27 34	26 93 04 06 04	10 78 28 01 28
80 78 85 54 10	39 77 09 33 17	18 27 02 11 41	52 08 57 80 93	85 63 88 11 40
02 39 57 75 85	08 85 42 35 62	33 54 44 57 96	10 97 64 47 21	24 78 10 96 90
73 72 07 01 84	69 11 27 18 87	80 14 07 28 76	38 98 42 34 09	63 95 74 47 51
61 38 31 72 22	55 23 41 89 63	39 00 69 35 04	18 27 34 98 15	52 80 26 96 75
39 16 14 42 82	14 97 59 74 98	25 97 33 36 05	60 14 13 00 30	77 67 74 92 94
80 90 77 44 84	39 88 41 98 85	37 31 10 42 09	49 67 53 95 96	01 05 24 39 99
09 05 26 56 70	63 66 03 40 35	06 57 88 78 37	28 12 54 88 83	50 48 25 57 35
33 42 52 42 26	32 04 36 00 82	20 41 88 50 47	53 57 03 25 54	64 09 95 23 26
72 65 16 94 74	72 64 87 15 30	55 45 41 95 76	15 55 22 05 77	12 12 45 00 38
04 14 23 20 92	83 58 66 18 25	35 48 23 27 36	63 40 39 14 99	37 19 60 27 62
85 22 61 41 93	52 21 36 07 46	24 41 45 78 58	31 88 45 12 66	82 29 37 35 53
54 88 80 35 79	91 67 45 95 02	08 61 93 37 90	29 01 18 51 93	62 26 22 86 84
33 25 85 15 16	82 03 24 52 33	39 35 13 32 29	59 46 46 55 45	76 80 91 69 82
75 97 31 59 57	32 40 58 20 81	02 21 45 71 43	33 52 64 71 94	94 52 67 32 53
90 63 87 53 14	35 38 13 44 51	49 24 61 14 65	25 10 27 14 89	89 88 39 97 08
65 06 11 54 98	93 34 83 35 66	19 42 76 68 26	03 04 49 73 61	08 15 94 74 85
64 42 00 74 27	82 23 39 78 12	39 57 20 77 66	65 84 42 99 80	15 53 39 01 14
27 17 51 73 89	76 96 37 51 17	45 58 09 99 04	47 16 05 53 64	25 66 62 54 05
32 21 53 00 94	87 27 65 68 96	15 62 53 25 94	80 66 30 80 82	19 42 28 07 17
54 20 95 80 43	72 35 08 98 28	02 70 61 68 15	89 98 53 73 80	44 03 25 93 66
59 28 66 69 64	84 84 37 15 49	67 55 33 38 67	83 01 16 62 13	69 37 22 39 03
83 87 25 81 67	01 22 19 55 58	22 19 66 59 05	38 78 50 13 55	47 48 92 81 70
83 31 05 70 80	03 36 68 00 17	39 60 14 06 98	56 43 46 40 55	02 49 55 08 80
64 54 52 95 00	13 35 17 86 47	92 62 81 93 54	60 47 95 73 38	52 13 30 71 14
39 26 90 00 76	55 48 90 15 24	76 56 82 25 71	20 32 88 46 23	30 18 84 39 04
29 76 30 56 75	28 19 36 55 14	11 95 47 85 99	63 90 22 13 46	19 21 99 02 86
06 31 00 29 98	01 46 32 77 38	90 28 81 76 97	64 51 13 95 52	34 69 40 32 11
97 54 14 76 07	57 65 55 91 02	21 85 14 44 46	07 97 65 42 95	84 67 17 87 55
82 96 88 57 17	11 61 99 77 21	53 51 35 37 81	98 94 13 84 01	70 93 91 13 19
76 87 83 47 27	12 52 49 06 42	16 92 11 36 69	17 42 08 44 83	68 30 98 52 41
87 39 47 88 84	87 23 79 20 86	95 63 36 68 41	22 90 66 49 89	86 95 25 47 00
74 04 01 27 31	59 61 63 36 97	12 59 24 48 91	67 98 27 29 72	89 79 51 05 87
47 89 64 14 13	66 43 17 00 46	50 79 34 59 20	96 56 46 79 58	56 36 94 47 25
87 77 87 16 97	64 14 85 43 63	92 11 43 40 37	59 06 16 20 51	62 04 93 35 26
96 97 76 31 43	72 21 98 00 40	11 99 04 76 98	95 62 17 29 90	29 04 78 58 93
43 82 01 32 85	77 81 18 16 97	29 93 77 07 50	02 02 93 23 77	00 55 68 66 87
57 20 38 39 60	40 09 63 92 34	65 95 35 99 11	91 41 15 55 73	88 42 74 55 73
49 06 57 21 71	80 93 90 60 17	90 32 36 36 87	07 93 29 95 87	49 01 42 64 52
94 25 08 42 70	95 79 81 34 77	80 13 92 63 35	55 16 97 34 17	40 76 64 51 70
68 14 88 13 82	82 38 31 86 74	40 45 39 28 28	30 04 23 74 12	43 42 34 51 38
12 20 89 78 09	33 61 92 88 63	41 64 61 67 34	88 86 03 26 36	41 98 58 46 15
88 31 78 97 05	26 11 91 24 16	19 43 86 56 65	60 98 95 97 66	11 41 81 82 50
56 72 88 03 59	29 61 36 30 52	15 25 14 46 84	64 68 14 23 54	51 02 97 76 80
07 13 81 23 20	01 07 31 93 04	87 04 25 89 20	28 45 48 10 69	93 97 86 66 59
21 18 86 45 54	55 61 83 60 88	24 33 18 43 90	16 02 21 22 00	77 55 97 56 61
02 15 41 22 50	88 73 84 39 17	03 65 52 10 99	60 80 56 32 46	26 84 23 58 16
90 95 38 52 38	32 77 10 73 05	36 18 14 74 20	19 68 13 31 84	41 38 60 32 49
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XVIII. IMMUNOLOGICAL TECHNIQUES

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1. PREPARATION OF COMMON MEDIA

1. Working TC 199 medium : Refer Chromosome preparation under Cytogenetics.

2. Working RPMI medium : Refer Sister chromatid exchange under Cytogenetics.

3. Hank's balanced salt solution (HBSS) : Reconstitute the commercial powder in 100 ml sterile distilled water and preserve it under 4°C. At the time of use dilute 1 : 10 and adjust the pH to 7.8 with 2% $NaHCO_3$.

4. Phosphate buffered saline (PBS) : Dissolve 1.21g K_2HPO_4, 0.34 g KH_2PO_4 and 8.0 g NaCl in 1 l of distilled water and adjust the pH to 7.3.

2. SEPARATION OF LYMPHOCYTES

To 6 ml of heparinised blood add 12 ml of PBS. Mix well by inversion. Take 3 ml of ficoll hypaque in a culture tube and carefully layer 6 ml of blood-saline mixture. Centrifuge at 400 g for exactly 30 min at room temperature. After centrifugation, carefully aspirate with a pasteur pipette the upper layer to within 0.5 cm of the opaque interphase containing mononuclear cells. Discard the upper layer.

Add to each tube 10 ml of 0.87% ammonimum chloride solution to lyse any contaminating erythrocytes. Allow to remain at room temperature for about 5 min. Centrifuge and discard the supernatant. Again wash with phosphate buffered saline and finally suspend the cell pellet in 0.5 ml of PBS. This will contain a cell population of more ahan 90% lymphocytes.

3. T—CELL ROSETTE TECHNIQUE

Reagents :

1. Hank's balanced salt solution (HBSS).

2. 10% Adsorbed fetal calf serum in HBSS.

3. 25% Glutaraldehyde solution.

4. Alsever's solution : Dissolve 2.05 g D-glucose, 0.42 g NaCl, 0.82 g trisodium citrate and 0.055 g citric acid in distilled water and make upto 100 ml. Sterilise and store in a refrigerator.

5. Preparation of sheep erythrocytes : Collect sheep blood aseptically into equal volume of Alsever's solution. Transfer 2.0 ml sheep blood into a tube in sterile conditions. Wash thrice with saline and discard the supernatant. Dilute the pellet to 0.5% with 10% fetal calf serum.

Procedure :

Collect 3 ml venous blood in a heparinised tube and keep it in an incubator for 45 min to settle. Take out the leucocyte rich plasma and wash 3 times with HBSS in cold. Resuspend in 1 ml of 10% fetal calf serum. To this add 1 ml of 0.5% sheep erythrocytes and incubate at 37°C for 20 min. Take out from the incubator and centrifuge. Keep the pellet at 4°C overnight. Resuspend the pellet in 1 ml HBSS. Add quickly 0.025 ml of 25% glutaraldehyde solution. Allow fixation in ice bath for 20 min. Wash the cells with HBSS once at 200 g for 5 min and resuspend in 1 ml of HBSS. Add 2 drops of 1% methylene blue and allow to stand at room temperature for 30 min. Make a coverslip preparation and count 200 consecutive lymphocytes to calculate the percentage of rosette forming lymphocytes. Lymphocytes with 3 or more of RBC adsorbed on the surface is counted as one rosette.

4. B—CELL ROSETTE TECHNIQUE

Materials :

1. Preparation of lymphocytes : Same as in the case of T-cell rosette technique.

2. Preparation of sheep erythrocytes : Isolation is same as in the previous procedure but finally a 5% suspension of the pellet is made in plain HBSS (without fetal calf serum).

Procedure :

Take 0.5 ml each of 5% sheep erythrocytes and 1 : 40 dilution of amboceptor in HBSS (rabbit anti sheep erythroctyte antibody) and incubate at 37°C for 30 min. Wash the cells in HBSS three times. Resuspend the cells in 0.5 ml HBSS. Add 0.5 ml of guinea pig serum (1 : 400 in HBSS). Incubate for 30 min at 37°C in water bath. Wash the cells in HBSS for 3 times. Resuspend the cells in 1 ml of HBSS. Mix with equal amount of lymphocyte suspension. Centrifuge at 200 g for 5 min and allow to stand at room temperature for 30 min. Shake vigorously and stain with 1% methylene blue as for T-cell rosette. Prepare coverslip preparation and count 200 consecutive lympohocytes. Cells with 3 or more RBC adsorbed on to the surface are counted as positive rosettes.

5. LYMPHOCYTE CULTURE

Principle :

The lymphocyte proliferation is assessed by measuring the DNA synthesis in terms of incorporation of radioactive thymidine into the cultured cells.

Reagents :

1. Phytohaemagglutinin (PHA).
2. TC 199 medium.
3. 3% Acetic acid.
4. 5% TCA.
5. Hyamine hydroxide.
6. Toluene scintillator : Refer Appendix.

Procedure :

Whole blood culture : Collect 3 ml venous blood in a sterile heparinised screw cap tube. Add 5 ml of TC 199 medium or RPMI in 4 sterile culture tubes. Add 0.4 ml of blood to each tube. The first 2 tubes act as controls and the 3rd and 4th as test. To the test add 0.1 ml PHA and incubate for 72 hr at 37°C. At the end of incubation add 0.1 ml tritiated thymidine to all the tubes and incubate for another 24 hr at 37°C. Terminate the culture by centrifuging and discarding the supernatant. Then wash twice with normal saline. Add 5 ml of 3% acetic acid to the pellet to lyse the RBC. Wash it once more with 3% acetic acid. Then wash 2 times with 5% cold TCA. Finally wash twice with cold methanol for 20 min. To the fine precipitate add 0.1 ml of hyamine hydroxide and keep in a water bath at 60°C for 30 min. Transfer to scintillation vials containing 10 ml toluene scintillator, keep it overnight and count.

Calculation :

$$\text{The ratio of thymidine incorporation} = \frac{\text{cpm in test culture}}{\text{cpm in control culture}}.$$

Note :

The method can be modified for smaller volumes of blood samples by using 0.2 ml of sample in 3 ml of culture medium.

Isolated lymphocytes (2×10^6/3ml of medium) can also be used to study the thymidine incorporation.

6. LEUCOCYTE MIGRATION INHIBITION

Principle :

Migration of leucocytes in the presence and absence of antigen is measured.

Procedure :

Collect 5 ml venous blood in a heparinised tube and keep it at 37°C in an incubator to settle RBCs. Aspirate the leucocyte rich plasma in a separate tube. Wash twice or thrice with TC 199 medium con-

taining 10% fetal calf serum. Finally resuspend the cells in the same medium at a concentration of 30 – 40 x 10^6 cells/ml. Fill the capillary tubes with cell suspension by capillary action. Seal the bottom with wax and centrifuge at 600 rpm for 2 – 3 min, cut the capillaries exactly at the cell-fluid interphase. Fix cell cotnaining portion in the four wells of a chamber with grease. In all four wells add medium and cover the first two with coverslips and seal with grease. In the 3rd and 4th wells add antigen along with the medium and seal. Allow them all to migrate at 37°C overnight.

Calculation:

Plot the area of migration on a graph paper. Measure the area by using a planimeter.

$$\text{Migration index (MI)} = \frac{\text{Average area of migration in chambers with antigen}}{\text{Average area of migration in chambers without antigen}}.$$

Note:

It is preferable to keep the chambers on a flat surface in a carbondioxide incubator.

7. MACROPHAGE MIGRATION INHIBITION FACTOR

Macrophage migration inhibition factor (MIF) can also be measured similarly by using peritoneal macrophages collected from guinea pigs.

Three days before the experiment 20 – 30 ml of paraffin oil is injected intraperitonially to induce peritoneal exudates in guinea pigs. The animals are sacrificed and the peritoneal exudate cells are collected by washing them with sterile Hank's balanced salt solution. The cells are taken in a separating funnel and remove excess of oil. Then the exudate cells are washed 3 times and finally suspended in TC 199 medium. From these take an aliquot, count the number of cells in a haemocytometer and setup the assay.

8. BACTERICIDAL ACTIVITY

Principle:

The bactericidal activity of leucocytes is assessed by incubating them in the presence of *E. Coli* and measuring the number of viable bacteria.

Reagents:

1. KRP buffer pH 7.4 : Refer Appendix.
2. Nutrient medium : Mix 1% beef extract, 1% peptone and 0.5% NaCl.
3. 2.6% Nutrient agar.
4. 5% Saponin.
5. *E. Coli* : Grow *E. Coli* in nutrient medium for 16 - 18 hr at 37°C. Then centrifuge and discard the supernatant. Wash the cells twice with saline and finally suspend it to a known volume. Standardise the number of *E. Coli* in this suspension by measuring the turbidity at 480 nm.

Procedure:

Collect venous blood (5 ml) into a heparinised sterile culture tube. After marking the volume, centrifuge the sample at 1200 rpm for 5 min. Transfer the plasma carefully into another culture tube. Wash the cells 3 times with 5 ml KRP buffer, centrifuge and discard the supernatants. Suspend the

washed cells to the original volume with KRP buffer and determine the WBC count.

Incubate 0.8 x 10^7 of polymorphonuclear neutrophil leucocytes and 1.6 x 10^7 *E. Coli* in 1.5 ml of KRP medium with 80% heat inactivated (56°C for 30 min) autologus plasma. After incubation at 37°C in a metabolic water bath for 60 min, 0.2 ml of the reaction mixture is mixed with 0.8 ml of 5% saponin to lyse WBCs. Prepare a control tube with the buffer, heat inactivated plasma and *E. Coli* and run similarly.

Count the number of viable bacteria by plating a suitably diluted aliquot on nutrient agar plates, by conventional colony counting technique. The viability is checked by trypan blue dye exclusion technique.

Calculation :

The bactericidal activity is expressed as $\log_{10}$ reduction in viable counts of *E. Coli* in the test when compared to the control at the end of 60 min.

Note :

Every week or fortnight a set of 3 - 4 plates of *E. Coli* must be cultured and the counts standardised.

For the assay the blood volume is adjusted with KRP buffer in such a fashion that during incubation the number of bacteria is double that of PMN leucocytes.

The processing of sample and dilutions must be carried out in a hood sterilised previously with a UV lamp.

References :

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2. Proc. Natl. Acad. Sci. **56**, 72 (1966).
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XIX. MISCELLANEOUS

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1. BILIRUBIN

Principle :

The coupling of bilirubin with p-benzenediazonium sulfonate to form azobilirubin is used for the estimation. Photometric estimation of the purple colour developed exactly 5 min after diazotisation with sulfanilic acid in aqueous solution is a measure of direct bilirubin. The colour developed after adding methanol at a level that releases bound bilirubin into solution without precipitating proteins represents total bilirubin.

Reagents :

1. Sulfanilic acid solution : Dissolve 100 mg sulfanilic acid in 1.5 ml conc. HCl and add water to make 100 ml.

2. 25% Sodium nitrite stock solution, store at 4°C. Prepare just before use by diluting stock sodium nitrite solution 1 : 50 with water (0.5%).

3. Diazo reagent : Prepare just before use by mixing 0.3 ml of 0.5% sodium nitrite solution with 10 ml of sulfanilic acid solution.

4. Diazo blank solution : Dilute 0.5 ml conc. HCl to 100 ml with water.

Procedure :

Take in two tubes 0.4 ml serum and dilute with 3.6 ml distilled water. To one (Blank) add 1.0 ml diazo blank solution and to the other (Test) add 1.0 ml dizo reagent and mix immediately. Read absorbance of Test exactly 5 min after addition of diazo reagent at 540 nm in a spectrophotometer against blank. And 5 ml absolute methanol to each tube and mix by gentle inversion. Let stand for 30 min and read the absorbance again at 540 nm, taking care to remove any air bubble trapped while transferring into the cuvette.

Calculation :

Assuming the absorptivity of azobilirubin equivalent to 5 mg bilirubin/100 ml at 540 nm to be 0.211, direct bilirubin (mg/100 ml plasma) = A_{540} at 5 min x 11.85, total bilirubin (mg/100 ml plasma) = A_{540} nm at 30 min after methanol x 23.70.

References :

1. J. Biol. Chem. 119, 481 (1937).

2. Clinical Chemistry, R. J. Henry, D. C. Cannon and J. W. Winkelman (Eds.), Harper and Row Publishers, 2nd Edn. p. 1051 (1974).

2. CARNITINE

Principle :

The spectrophotometric assay is based upon the premise that carnitine present will permit the liberation of an equimolar amount of free CoA under conditions in which the initial concentrations of acetyl

CoA, carnitine acetyl transferase and 5,5' - dithiobis (2-nitrobenzoic acid) are held constant. The combination of DTNB with sulfhydryl groups forms the thiophenolate ions which absorbs at 312 nm ($E_{max} = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$).

Reagents:

1. 1 M Tris-HCl, pH 7.5 : Dissolve 12.1 g tris in about 65 ml of water, adjust the pH to 7.5 with HCl and make up the volume to 100 ml with water.

2. 5, 5' - Dithiobis (2-nitrobenzoic acid, DTNB) : Dissolve 1.980 mg of DTNB in 5 ml of tris-HCl buffer pH 7.5.

3. Acetyl coenzyme A : Dissolve 1.215 mg acetyl CoA (commercial preparation) in 3 ml water.

4. Carnitine acetyl transferase-CAT (5 mg protein/ml) : Take 0.90 ml of glass distilled water in a clean test tube, add 0.10 ml of CAT (commercial preparation - Sigma) and shake well.

5. Perchloric acid : Dilute 4.2 ml of 70% perchloric acid to 100 ml with glass distilled water.

Reagents 2, 3 and 4 should be prepared fresh.

6. Standard : Prepare standard by dissolving 1.977 mg carnitine hydrochloride (Sigma) in 100 ml glass distilled water. This gives a concentration of 10 n moles/0.1 ml.

Procedure :

Pipette out 1 ml plasma into a clean test tube and add equal volume of perchloric acid solution. Mix well, keep for 10 min and centrifuge for about 15 min at 2500 rpm in a clinical centrifuge. Take a known quantity of the supernatant fluid and neutralise it to pH 7.0. This neutral extract can be used for the estimation of free carnitine. Carnitine is stable for several days in the neutral extract at 15°C.

Assay system : Pipette into clean dry cuvettes the following regeants :

Reagent	Blank (ml)	Standard (ml)	Sample (ml)
DTNB	0.1	0.1	0.1
Acetyl CoA	0.1	0.1	0.1
Standard	0.1	0.1	—
Sample	0.2	—	0.2
Water	0.59	0.69	0.59
	Incubate at 37°C for 10 min and measure OD (Eo).		
Enzyme	—	0.01	0.01
Water	0.01	—	—
	Mix well and measure OD at 5 min (E_1).		

It is necessary to determine in a separate cuvette the amount of absorbancy change that occurs when only DTNB and enzyme are added (E_2). This enzyme blank must be subtracted from E_1.

Plot the absorbance of the standard graphically and read the carnitine concentration of the test from the graph and multiply the results obtained by the appropriate dilution factors.

Note :

A serious source of error is inconsistancy of measuring temperature and so temperature control of the cuvette holder is necessary. This method is slightly influenced by pH changes. The pH must not exceed 8.5 since the enzyme (CAT) is otherwise inactivated, the pH should not fall below 7.0, since insufficient dissociation of the dye DTNB would then lead to low results.

References :

1. J. Lipid Res. **6**, 184 (1964).

2. Methods of Enzymatic Analysis. H.U. Bergmeyer (Ed.), Academic Press Inc. New York and London, Vol. **4**, p. 1762 (1974).

3. URIC ACID

Principle :

Uric acid reduces phosphotungstate to tungsten blue which is measured photometrically.

Reagents :

1. Protein precipitant-tungstic acid solution : To 800 ml of water add, with mixing, 50 ml of 10% sodium tungstate and 0.05 ml of 85% orthophosphoric acid and 50 ml of 2/3N H_2SO_4. Mix well and store in poltyhene bottle (discard if cloudy). Stable for months.

2. Phosphotungstic acid : Dissolve 14 g molybdenum free sodium tungstate dihydrate in about 50 ml of water in a one liter reflux flask. Add 11 ml of 85% orthophosphoric acid and mix. Add few glass beads. connect to a reflux condenser and reflux gently for 2 hr. Cool to room temperature. Add about 50 ml water and 11 g of lithium sulphate ($Li_2 SO_4.H_2O$) mix until dissolved. Dilute to 1 l with water. The reagent is stable for at least 2 years, when stored in the cold.

3. 10% Na_2CO_3 : Dissolve 10 g Na_2CO_3 (anhydrous) in water and dilute to 100 ml. Stable for at least 2 years when stored in polythene bottle.

4. Uric acid (62.5 mg/100 ml) : Dissolve 60 mg of Li_2CO_3 in about 50 ml of water in a 100 ml volumetric flask using gentle heat (do not allow temperature to exceed 50°C) and mixing. Add exactly 62.5 mg uric acid and continue to stir until completely dissolved. Add 2 ml of formaldehyde and 0.15 ml of glacial acetic acid. Mix and dilute to 100 ml with water. Stable for 2 years when stored in the cold.

Working standard (10 μg/ml) : Dilute 0.4 ml of stock to 25 ml with distilled water.

Procedure:

Take 1 to 5 ml of working standard and make to 5 ml with water Add 1 ml of 10% Na_2CO_3. Let stand for 10 min. Add 1 ml of phosphotungstic acid reagent. Mix immediately. Allow colour to develop for 30 min and take readings within 20 min at 700 nm on spectronic-20.

Add 0.5 ml of plasma or serum to 4.5 ml of protein precipitant. Filter and take 2–2.5 ml of protein free filtrate. Make to 5 ml and proceed as described for standards.

Calculation :

Read the unknown from the standard graph and express as mg uric acid/100 ml.

Note :

For urine, if cloudy, warm a portion of the well mixed urine at 60°C for 10 min to dissolve any precipitated urates. Dilute 1 ml to 100 ml with water and use 5 ml for estimation as described for protein free filtrate for serum.

Reference :

Standard Methods of Clinical Chemistry. D. Seligson (Ed.), Academic Press Inc, Publishers, New York and London, Vol. IV, p. 239 (1963).

4. HIPPURIC ACID

Principle :

Hippuric acid in urine is extracted and then estimated in terms of nitrogen by the conventional Kjeldahl procedure.

Reagents :

1. Sodium hypobromide : Prepared by mixing equal volumes of 25% NaOH and a solution containing 12.5 g bromine and 12.5 g of sodium bromide dissolved in 100 ml of water.
2. N/50 and 5 N H_2SO_4.
3. 0.1% Methyl red indicator in alcohol.

Procedure :

The original method has been slightly modified. Instead of an extraction tube and 500 ml Kjeldahl flask assembly used in the original method quickfit liquid–liquid extractor is used for extracting hippuric acid from urine.

Urine 20 ml and 1 ml of concentrated HCl are placed at the bottom of the long extractor tube and hippuric acid is extracted from it with diethyl ether for about 6 hr. The flask is filled with enough ether and it is then connected with the side arm of the extractor tube. The flask containing ether is heated on electric bulb. The top ether layer in the extractor tube is then transferred to the flask by tilting the extractor tube, care being taken not to disturb the bottom urine plus water layer. The ether in the flask is then quantitatively transferred to a 250 ml Kjeldahl flask and it is distilled off. To the dry residue is then added 5 ml of hypobromide solution and mix well. This is followed by addition of 2 ml of H_2SO_4 (1:5), 2 ml of 25% NaOH and 2 ml of hypobromide solution, with thorough mixing after each addition The nitrogen of the residue is then determined by conventional Kjeldahl method (Refer Proteins, aminoacids and metabolites).

Calculation :

1 ml of N/50 H_2SO_4 = 0.28 mg of Nitrogen.

Reference :

J. Biol. Chem. 69, 197 (1926).

5. OXALIC ACID

Principle :

The oxalic acid is extracted from foodstuffs and precipitated as calcium oxalate which is then titrated against standard potassium permanganate.

Reagents :

1. 2 N HCl.
2. Phosphoric–tungstate reagent : To 24 g of sodium tungstate dissolved in water is added 40 ml of phosphoric acid and the solution is diluted to 1 l with water.
3. Calcium chloride buffer : To 25 g calcium chloride dissolved in 500 ml of 50% glacial acetic acid, is added 330 g of sodium acetate dissolved in 500 ml of water. The two solutions are then mixed well.
4. 0.1% Methyl red.
5. N/100 $KMnO_4$.

Procedure :

Extraction of the sample : To 5 g of well ground sample, is added 100 ml of 2 N HCl and the mixture is shaken well for about 2hr in a mechanical shaker. It is then centrifuged and filtered (in the case of fresh sample, 10 g of the sample is well ground in a mortar along with 25 ml of 2 N HCl). The mixture is transferred to the same beaker and weighed. It is then boiled for about 15 min and cooled. The mixture is adjusted to the previous weight with distilled water, made upto 100 ml with 2 N HCl, shaken well and filtered.

To 25 ml of the filtrate is added 5 ml phosphoric–tungstate reagent, stirred well and kept overnight. The next day, it is centrifuged and filtered. To 20 ml of the filtrate is added 2 or 3 drops of methyl red, neutralised with ammonia, 5 ml of calcium chloride buffer is added and stirred well. The mixture is allowed to stand overnight at the end of which it is filtered through Whatman No. 40 or 44 filter paper and washed free from chloride using distilled water (silver nitrate test). The precipitate along with the filter paper is transferred to the same beaker and some distilled water is added followed by 5 ml of 2 N H_2SO_4. The mixture is heated to 80°C over a burner and titrated against N/100 potassium permanganate solution.

Caculation :

1 ml of N/100 $KMnO_4$ = 0.45 mg of oxalic acid.

$$\text{mg Oxalic acid/100 g sample} = \text{Titre value} \times N \text{ of } KMnO_4 \times \frac{0.45}{0.01} \times \text{dilution factor} \times \frac{100}{\text{Wt of sample}}$$

Reference :

The Analyst, **77**, 340 (1952).

6. QUINOLINIC ACID

Principle :

Quinolinic acid is converted into niacin by acid hydrolysis. By estimating the niacin content before hydrolysis and after hydrolysis, the quinolinic acid content in the sample can be determined.

Reagents :

Reagents for microbiological assay of niacin : Refer Vitamins, coenzymes and metabolites.

Procedure :

Filtered urine sample 5 ml is taken in a porcelain dish and is evaporated to complete dryness. The residue is dissolved in 5 ml of glacial acetic acid. Acetic acid solution 2.5 ml is taken in a test tube and autoclaved at 15 lb for 1 hr. After hydrolysis the pH is adjusted to 6.8 and the volume is made upto 40 ml. The niacin content in aliquots of the sample is estimated microbiologically as described earlier.

Calculation :

The value obtained will give the total amount of niacin and quinolinic acid. By subtracting the niacin value from this and muliplying the result by a factor of 1.67, the quinolinic acid content in the sample is obtained.

References :

1. J. Biol. Chem. **181**, 667 (1949).
2. J. Biol. Chem. **193**, 627 (1951).

7. NON-PROTEIN NITROGEN

Principle:

Proteins in blood are first precipitated by Folin's tungstic acid reagent. The nitrogen content in the filtrates is determined by micro –Kjeldahl procedure.

Reagents :

1. 10% Sodium tungstate.
2. 2/3 N H_2SO_4.
3. Reagents for micro-Kjeldahl procedure for estimation of protein (Refer Proteins, amino acids and metabolites).

Procedure :

Blood 1.0 ml is dropped into 7 ml of water and add 1.0 ml 10% sodium tungstate and mix. Add 1.0 ml of 2/3 N H_2SO_4 slowly with shaking. After 10 min, the mixture is filtered into a dry container.

If the colour of the mixture does not change to brown on addition of H_2SO_4 and the supernatant is not clear at the end of 10 min, 1 or 2 drops of 10% H_2SO_4 may be added. If the mixture does not turn colour even by this treatment, the sample will have to be rejected. If serum or plasma is used, 8 ml of water and 0.5 ml each of sodium tungstate and H_2SO_4 are added.

Take in a 50 ml volumetric flask, 25 ml of water and 5.0 ml of milk are added dropwise with shaking. Add 0.5 ml of tungstate followed by 1.0 ml of 2/3 N H_2SO_4 and mix well. The volume is then made up to 25 ml and left aside for 30 min. The mixture is then filtered.

Pipette out 5.0 ml of the filtrate (blood, serum, plasma or milk) into a micro - Kjeldahl digestion flask. The nitrogen is then determined as described under total proteins. A reagent blank is carried out with 5.0 ml of the reagents alone.

Note:

The nitrogen present in all the constituents of the blood which are not precipitated as protein is called non-protein nitrogen. Included in this are, urea, uric acid, creatinine, amino acid and other such known and unknown compounds.

Reference:

Microtechniques of Clinical Chemistry. S. Natelson (Ed.), Charles C. Thomas Publishers, 2nd Edn, p. 315 (1961).

8. FIBRINOLYTIC ACTIVITY

Principle:

When plasma is diluted and acidified the precipitate which form contains plasminogen activator, plasminogen and fibrinogen. The natural inhibitors of fibrinolysis are not precipitated. The precipitate is redissolved, the fibrinogen is clotted with thrombin and the time for clot lysis is estimated. The test measures predominantly plasminogen activator activity.

Reagents:

1. 3.1% Trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) solution.
2. 0.85% NaCl.
3. Veronal acetate buffer, pH 7.4 $\pm$ 0.05.
4. Bovine thrombin : 3000 NIH units diluted in 15 ml of normal saline to make 200 U/ml solution. This may be stored at 0°C for periods upto 2 months and may be diluted further with saline.
5. 1% Glacial acetic acid.

Procedure:

Processing of blood sample : Using clean, dry, siliconised syringes and needles, free flowing venous blood (ie. without the application of torniquet, pressure cuff etc.) is collected into siliconised ice cold centrifuge tubes containing 3.1% sodium citrate solution (about 1/9th of the volume of blood)

immersed in a cooling mixture of ice and salt. The temperature of blood or plasma should be maintained at about 0°C, never above 5°C till the time of clotting. The blood is centrifuged (within 30 min from the time of collecting the blood) below 4°C for 5 min at 1500 rpm and as much plasma as possible is separated from this. This plasma is rich in platelets.

To 19 ml of cold distilled water, 0.32 ml of 1% acetic acid and 1 ml of plasma are added. On stirring at room temperature, a white precipitate settles down. The mixture is centrifuged at 0°C at 1800 rpm for 15 min. The supernatant is discarded and the precipitate reconstituted in 1 ml of veronal buffer.

To 0.3 ml of this euglobulin solution, 0.05 ml of thrombin solution (200 U/ml) is added and immediately incubated in a beaker of water in an incubator at 37°C. The time taken for the complete disappearance of the clot (till no white patricles are visible) at 15 min intervals upto 12 hr is noted and the observations are continued the next day if necessary. The lysis time is, the euglobulin clot lysis time (ELT).

Note :

ELT is inversely correlated with the fibrinolytic activity of blood in a general sense but it is more specifically correlated in an inverse manner only with the fibrinolytic activity of the euglobulin fraction of the plasma.

Reference :

Practical Haematology, J. V. Davice and S. M. Lewis (Eds.) Churchill Livingstone, 5th Edn. p. 367 (1975).

9. BASAL METABOLIC RATE

Principle :

The basal metabolic rate (BMR) is expressed in terms of heat production per unit surface area. It is measured with the instrument—Benedict-Roth respiration apparatus.

(i) Benedict-Roth respiration apparatus :

The apparatus consists of a bell type spirometer, kymograph drum, a timer and a mouth piece with attachments.

Bell type spirometer : This is a hollow double walled cylindrical vessel. In a narrow space between the two walls fits a second inverted hollow cylinderical vessel or bell. The bell is counterpoised so that it rides easily up or down in the annular space between the two walls. Inside this, there is a can containing the absorbant soda lime (for the absorption of the CO_2 in the expired air). This has a hole at the bottom which is covered by a dish-shaped screen and the tube carrying the expired air is fitted into this. The annular space between the two walls contains water which acts as a seal. The spirometer bell has a volume of 20.73 ml for every mm of height. With a bell of this size each mm fall of the bell indicates heat production in terms of calories which is equal to one kilocalorie per hr.

Kymograph drum : The kymograph sheet is fixed on the revolving drum and this also is mounted on the same metallic platform on which the spirometer is fixed,

A small pen attached to the spirometer bell rests on the kymograph sheet fixed on the revolving drum. These are calibrated so that the mesurements of heat production can be made directly in terms of calories by taking into consideration the volume of the spirometer.

It is important that the drum should be securely mounted on its shaft. Care should also be taken against the possibility of the paper slipping on the drum while the tracing is being taken. It should be fastened on the drum at some convenient point with a bit of gum or adhesive tape.

Timer : Underneath the platform on which the spirometer and kymograph drum are fixed, is the timer. This is attached with the kymograph drum in such a way that when the timer is started the drum starts revolving at such speed that it completes one vertical column on the kymograph sheet in 1 min. In case this does not coincide, the speed of the revolving drum can be adjusted by means of a small knob near the base.

Absorbant : It is one of the essential parts of the apparatus and demands continuous attention. The soda lime used should not be too coarse nor too fine (granules of the size of a pea are the best). It is very essential to see that the sodalime used is efficient in absorbing carbondioxide from the expired air and should be changed frequently.

The accuracy of the BMR determination depends on the efficiency with which the CO_2 in the expired air is absorbed. Usually, increased amplitude and rate of respiration during a test indicate accumulation of CO_2 in the circulating air due to an exhausted absorber or insufficient ventillation.

Mouth piece : It is a rubber piece attached to a metallic piece, the other end of which is connected to the rubber tubes. It is oval shaped, consists of two lugs and fits into the mouth of the subject. After use, the rubber mouth piece should be washed thoroughly with soap and sterilised. Disinfectants which are likely to leave residual taste on the rubber should be avoided.

Two tubes are connected to the metal mouth piece assembly. One tube is connected to the spirometer through which the oxygen is supplied to the subject undergoing the test. The other tube is attached to the can containing sodalime through which the expired air passes. Both the tubes work one way and are controlled by flutter valves. Care should be taken to see that the tube carrying expired air is kept dry by draining off the condensed water.

Thermometer : This is a clinical thermometer attached to the spirometer. Temperature should be recorded at the beginnig and also at the end of the test.

(ii) Preparation of the subject :

Preparation of the subject needs great care. The entire success of the test depends primarily on securing and maintaining basal conditions. The subject should be mentally as well as physically at complete rest. Surroundings also play an equally important part. A quiet place is chosen for carrying out the test.

The subject should be instructed to have only a light meal on the previous evening, 10 – 12 hr before the test is carried out. It is preferable for the subject to sleep in the same room where the test is conducted, in order to avoid any strain on the subject. In case, this is not possible, the subject may be brought to the metabolic unit as soon as he wakes up from sleep and is allowed to rest in bed at least for an hr to bring him to complete basal conditions.

(iii) The test :

Before starting the actual test, make sure that the spirometer is filled with oxygen to raise the spirometer bell. Fill the pen with ink and allow it to rest on the kymograph sheet fixed to the revolving drum.

During the test the subject lies down quietly and familiarises himself with the mouth piece. The mouth piece should be inserted by the investigator or the subject himself be allowed to insert it, if desired. The nose clip is applied and the subject is instructed to breathe slowly, through the mouth. After a few min, when the subject's breathing becomes regular, connect him to the apparatus by turning the knob attached to the metallic piece to which the tubes carrying the gases are attached. Again, wait for sometime before starting the actual test to allow uniform conditions to be established not only in the apparatus but also in the subject. Turn the knob which operates the timer. During the test the spirometer bell raises up and down with the respiration of the subject and these will be traced on the kymograph sheet. Continue recording for 8 min. At the end of 8 min, stop the timer and disconnect the subject from the apparatus. If necessary introduce a fresh lot of oxygen and repeat the test for another 8 min. Take the mean value of the 2 readings for calculation. If there is a difference of more than 5% between the two readings, repeat the test for the third time and take two closely related values for calculation. Take height and weight of the subject at the end of the test.

Calculation :

Calculate the surface area from the nomogram given on the kymograph sheet or using the formula, $A = Wt^{0.425} \times Ht^{0.725} \times 71.84$ where, A is the surface area in sq. cm, Wt is the weight in Kg and Ht is the height in cm.

Note the barometric pressure and temperature. From the temperature and pressure noted down the correction for these factors is determined by reference to the table given on the kymograph sheet.

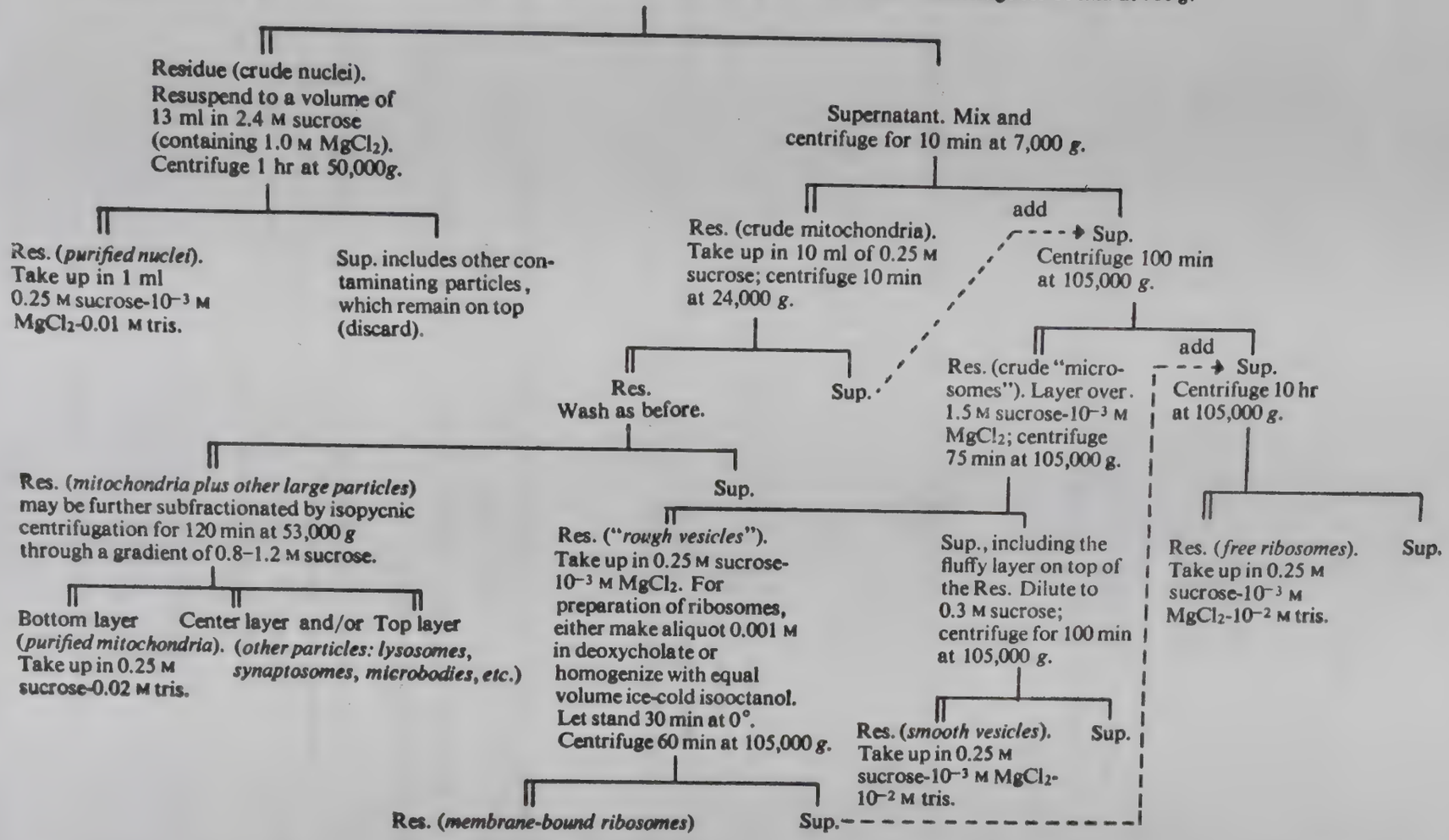
$$\text{BMR in Cal/m}^2\text{/hr} = \frac{\text{Calories from kymograph sheet x correction for temperature and pressure.}}{\text{Surface area}}$$

Reference :

Hawk's Physiological Chemistry, B.L. Oser (Ed,), Macgraw – Hill Book Company, 14th Edn. p. 922 (1965).

10. A TYPICAL CELL FRACTIONATION PROCEDURE

Homogenize minced tissue in 0.32 M sucrose, containing 0.02 M tris, *p*H 7.6, 3×10^{-3} M $MgCl_2$, to prepare a 25 per cent homogenate (1 part tissue, 3 parts medium). Filter through cheesecloth; dilute each 12.5 ml to a final volume of 20 ml containing sucrose at 0.25 M. Layer an equal volume of homogenizing medium underneath. Centrifuge for 10 min at 700 *g*.



Reference : Biological Chemistry, H. R. Mahler and E. H. Cordes (Eds.), A Harper International Edn. p. 394 (1969).

11. DENSITY GRADIENT CENTRIFUGATION TECHNIQUE FOR SYNAPTOSOMES

Principle :

The crude-mitochondrial (P_2) fraction of rat brain isolated at 17,000 g by differential centrifugation, mainly consists of myelin, synaptosomes and mitochondria. Since these particles have almost identical sedimentation characteristics, further separation into individual components is only possible by density gradient centrifugation technique. In principle, particles having identical sedimentation characteristics, when layered onto a suitable density gradient medium, would migrate to their corresponding isopycnic density under centrifugal force. The exact requirements of force and time of centrifugation have been worked out for separation of P_2 subfractions of rat brain. For generating the density gradient, several natural and synthetic polymers such as sucrose, dextran, ficoll etc. are in use. Employing a discontinuous gradient of 0.8 to 1.2 M sucrose, a procedure is described here for the separation of P_2 subfractions of rat brain.

Reagents :

1. Prepare the following solutions of sucrose (Sigma grade) in glass distilled water. Solutions should be prepared fresh on the day of use.

(i) 0.32 M Sucrose : 109.5 g in 1 l, (ii) 0.80 M sucrose : 273.8 g in 1 l and (iii) 1.20 M sucrose : 410.8 g in 1 l.

2. Prepare the gradient by slowly layering with a pipette 2.5 ml of 0.8 M sucrose over 2.5 ml of 1.2 M sucrose in 5.5 ml capacity cellulose nitrate tubes (Spinco, Beckman). These tubes are supplied for use in swinging bucket rotor type SW 50 L. For other types of rotors, corresponding volumes of these two sucrose solutions should be used. Leave the gradient at 0-4°C for 1 to 2 hr.

Procedure :

Sacrifice albino rat by decapitation, remove the brain quickly, wash it with cold normal saline and place it in beakers containing 0.32 M sucrose. Keep them in ice.

Prepare a 5 or 10 per cent homogenate of the brain in cold 0.32 M sucrose at 100 rev/min using 5 or 6 complete up and down strokes.

Centrifuge the homogenate at 800 x g for 10 min in a refrigerated centrifuge using swing out rotor. Transfer the supernatant (supernatant-1) carefully. Resuspend the sediment in 5 or 10 ml of 0.32 M sucrose and centrifuge at 800 x g for 10 min. Transfer the washings to supernatant-1 and combine. The sediment is designated as nuclear pellet (P_1).

Centrifuge the combined supernatant-1 fraction at 17,000 x g for 60 min in a fixed angle rotor either in a refrigerated centrifuge or ultracentrifuge. Discard the supernatant (supernatant-2) and resuspend the sediment in 5 or 10 ml of cold 0.32 M sucrose and centrifuge at 17,000 x g for 60 min. The supernatant (washings) can be discarded. The sediment here is designated as crude-mitochondrial fraction (P_2).

Suspend the P_2 pellet slowly and gently in cold 0.32 M sucrose (2 to 3 ml per g of original tissue).

Layer 0.5 ml of P_2 suspension over the sucrose gradient carefully. Place the tubes in the SW 50 L rotor and centrifuge at 53,000 x g for 2 hr or the gradient centrifugation may be carried out at 100,000 x g for 30 min. This modification of Whittaker's original procedure is beneficial in reducing the time of operation and also is reported to aid better separation of the subfractions of P_2.

The P_2 fraction clearly separates into 3 distinct layers which are (from top to bottom), A-small myelin, B-synaptosomes and C-mitochondria. Using a syringe with a bent needle, carefully aspirate out the layer A and then proceed to collect the layer B in a separate tube. If it is desired to sediment layer B, slowly dilute the fraction with an equal volume of glass distilled water before centrifuging at 17,000 x g for 60 min. This fraction is designated as synaptosomal fraction.

Reference:

Methods of Neurochemistry. R. Fried (Ed.), Marcel Dekker INC, New York, Vol. 2, p. 1 (1972).

12. POLYACRYLAMIDE GEL ELECTROPHORESIS

Principle:

When a mixture of high molecular weight compounds is allowed to traverse by applying electric current through the gel matrix of a polymer of acrylamide various components get resolved based on the charge. The method has high resolution capacity which is further improved by the use of sodium dodesyl sulfate which in addition allows the resolution on the basis of molecular weights of the proteins.

Complete polyacrylamide gel electrophoresis (PAGE) equipment with high purity reagents can be procured from Canalco Co., U. S. A.

Reagents:

1. 0.2 M Na-Phosphate buffer pH 7.2 : Dissolve 8.89 g of $NaH_2PO_4.2.H_20$ and 25.65 g of $Na_2HPO_4\ 2H_2O$ and make upto 1 l with glass distilled water. Depending on the brand and batch of phosphate it is not unusual to find slight variations in these proportions. Deaerate the buffer by application of vacuum in a conical flask.
2. Acrylamide solution : Dissolve 22.2 g acrylamide and 0.6 g methylene bis acrylamide in water and make upto 100 ml with glass distilled water. Filter through Whatman No. 1 filter paper.
3. Ammonium persulfate : 15 mg/ml in water.
4. N, N_1 N', N' – Tetramethylethylene diamine (TEMED) solution.
5. Bromophenol blue (Tracking dye) : 50 mg in 100 ml water.
6. Coomassie blue (Staining dye) : 0.6 g in 250 ml of 50% methanol.
7. Destaining solution : Mix 454 ml of 50% methanol and 46 ml of glacial acetic acid.

Procedure:

Acrylamide gel 10% for a typical run of 12 gels. Mix well 15 ml of deaerated phosphate buffer, 13.5 ml of dearated acrylamide solutions, 1.5 ml of ammonium persulfate solution (freshly prepared) and 0.045 ml of TEMED solution.

Arrange the clean and dry gel tubes (100 x 6 mm, vertically. Fill each tube with 2 ml of the above solution. If available, gels could be held in front of a fluoroscent light at about 1 foot distance. Before the gel hardens layer a few drops of water on top of the gel solution. After 10-20 min an interface could be seen indicating that the gel has solidified. Just before use the water layer is removed and the tubes placed in an electrophoresis apparatus.

Mix tracking dye 3 μl and 10-50 μl protein solution containing 20-50 μg protein. Apply this to the top of the gel. Dilute gel buffer 1 : 1 with water and layer it carefully on the top of each tube to fill the tubes. The two compartments of the electrophoresis apparatus are filled with the diluted buffer.

Apply a constant current of 5 mA or 8 mA per gel with the positive electrode in the lower chamber.

Under these conditions tracking dye moves about three fourth the tube length in about 4 hr. The time taken to run the gel may be decreased by decreasing the molarity of gel buffer.

After electrophoresis, the gels are removed from the tubes by pushing water from a syringe between gel and glass wall. This requires some practice to successfully bring out the gels without breaking. The length of the gel and the distance travelled by the dye are measured.

Place the gels in small separate tubes filled with staining dye solution (Coomassie blue) for 2-10hr at room temperature.

For destaining remove the staining dye, rinse excess dye repeatedly with glass distilled water and place in destaining solution for half an hour. The destaining now can be continued by changing the destaining solution from time to time or electrophoretically in 2 hr in the same electrophoresis apparatus using destaining solution.

The gels can be stored in 7.5% acetic acid.

Note :

The above procedure is a typical one with 10% acrylamide gel. There are several variations of this proeurecd with the same basic requirements eg. instead of 10%, 5% or 7.5% acrylamide can be used depending on the best resolution obtained for the system under study.

The procedure can include-SH stabilising agents like mercaptoethanol or can be carried out in the presence of 1% sodium dodesyl sulfate useful for molecular weight determination.

Reference :

J. Biol. Chem. **244**, 4406 (1969).

13. PURIFICATION OF WATER

Principle :

Water is the most important and widely used item in the laboratory. The degree of error caused by impurities in water is directly proportional to the sensitivity of the methodology. The usual methods employed in the laboratories for the purification of water are, distillation and deionisation.

Distillation :

This, although the oldest method, is still the most commonly used method for water purification in most of the laboratories. In view of this, it is essential that the research worker be aware of its limitations, which are (a) only non-volatile materials are removed; liquids with boiling points near 100°C as well as some organic compounds (that can be steam-distilled) with higher boiling points contaminate the distilled water, (b) tiny droplets of water with all its impurities cross over to the receiver because of rapid agitations within the still; installation of baffles minimises the problem but does not completely eliminate it and (c) the residual non-volatile material in the still may lead to problems.

Deionisation :

Charged species are removed by mixed ion exchange resin beds through which water is passed. This process however (i) does not remove organic materials, particles and micro-organisms and (ii) can only handle water that has already undergone a preliminary purification.

Ideal water purification system : An ideal water purification system that provides "Reagent Grade" water is now replacing the older distilled or glass distilled water provided by distillation. Systems of this type now include (i) a reverse osmosis unit for primary clean up (ii) an activated charcoal cartridge for the removal of organics (iii) one or two mixed-bed deionisation cartridges for the removal of inorganics and (iv) a filter to remove particulate matter including bacteria.

The deionised (demineralised) water is obtained by using the deionisation unit. The deionisation unit comprises a small pressure vessel, a control panel, a collapsible regenerant container and supporting framework. The pressure vessel contains an intimately mixed bed of cation exchange and anion exchange materials.

As raw water passes down through the bed Ca^{++}, Mg^{++} and Na^{+} ions associated with the neutral and alkaline salts in the water are exchanged for hydrogen ions (H^{+}) by the cation exchanger resulting in the formation of the equivalent acids. These acids together with any silica and free CO_2 are exchanged by the anion exchange material for hydroxyl ions (OH^{-}). Thus the original dissolved solids content of raw water is replaced with H^{+}, OH^{-} or H_2O-water. Because the ion exchange materials are intimately mixed the water is subjected during service to a large number of demineralising stages thus producing water of very high purity. After demineralising a certain quantity of water the exchange capacity of the ion exchange materials becomes exhausted. To restore this capacity they must be regenerated. The anion exchanger is regenerated with NaOH and the cation exchanger with HCl. The need for regeneration is shown by the conductivity tester.

14. PURIFICATION OF THE COMMON ORGANIC SOLVENTS

a. Absolute ethyl alcohol

Transfer about 2 l of rectified spirit (95% ethyl alcohol) into a 3–4 l distillation flask. Add about 500 g quicklime which has been heated in a muffle furnace (300–400°C) immediately before use. Fit the flask with a condenser carrying a drying tube filled with glass wool or anhydrous calcium chloride. The mixture is gently refluxed for 6 hr and allowed to stand overnight. Distil the alcohol the next day. The receiver is fitted with a special adapter to which is attached a drying tube containing acid washed glass wool or anhydrous calcium chloride. The alcohol obtained in this way is 99.5% and very hygroscopic, and the bottle should be closed by a well fitting ground glass stopper or by a tightly fitting rubber stopper.

b. Absolute diethyl ether

The chief impurities in commercial ether are water, ethyl alcohol and in samples which have been exposed to the air and light for sometime, ethyl peroxide. (The presence of peroxides may by detected by the liberation of iodine, brown or blue colouration with starch solution, when a small sample is shaken with an equal volume of KI and a few drops of dilute HCl).

Remove the peroxide by shaking with a concentrated solution of 10-20 ml of ferrous sulfate solution (prepare from 60 g of ferrous sulphate and 6 ml of concentrated sulphuric acid in 110 ml of water) to 1 l of ether. Remove the aqueous phase and transfer ether into a 2 l flask. Treat the ether with 150 200 g of anhydrous calcium chloride and allow to stand for 24 hr with occasional shaking. This treatment removes water and the alcohol. Filter the ether through a large fluted filter paper into another dry bottle. Introduce sodium wire (7g) into ether. Close the bottle and allow to stand for 24hr. If on the following day no bubbles of hydrogen rise from the sodium in the ether and the latter still possesses a bright surface the bottle is closed tightly and kept in a cool place. If, however, the surface of the sodium wire is badly attacked due to insufficient drying with the calcium chloride, the ether must be filtered through a fluted filter paper into another clean and dry bottle and sodium treatment repeated. If a fresh supply of high grade ether or of analytical reagent quality is available, the treatment with ferrous solution may be omitted. Ether is highly inflammable and extremely volatile and great care should be taken that there is no naked flame in the vicinity of the liquid.

c. Absolute methyl alcohol

Place 5 g clean dry magnesium turnings and 0.5 g resublimed iodine in a 2 l distillation flask fitted with a reflux condenser. Add 50–75 ml of methyl alcohol through the condenser and the mixture warmed until iodine disappears. If a vigorous evolution of hydrogen does not take place add a further 0.5 g of iodine and treat the mixture until all the magnesium is converted into the methoxide. About 900 ml of methyl alcohol are then added and then boil the mixture for 30 min under reflux. Distil the product with exclusion of moisture discarding the first 25 ml of distillate.

Acetone present in synthetic methyl alcohol can be removed when present in quantities upto one percent. Reflux a mixture of 500 ml of alcohol, 25 ml of furfural and 60 ml of 10% NaOH solution for 6–12 hr followed by distillation. The resin formed carries down all the acetone.

d. n-Propyl alcohol

Dry the purest commercial n-propyl alcohol with anhydrous potassium carbonate or anhydrous calcium sulfate and distil collecting the fraction boiling between 96.5–97.5°C/760 mm. If perfectly dry alcohol is required treat with magnesium activated with iodine (See Absolute methyl alcohol).

e. iso-Propyl alcohol

Two technical grades marketted are 91% and 99% respectively. The former has a boiling point of about 80.3°C and is a constant boiling mixture with water. Remove water with excess of freshly burnt quicklime and distil from the lime (See absolute ethyl alcohol).

f. Acetone

Reflux commercial acetone with successive small quantities of potassium permanganate until the violet colour persists. Later dry with anhydrous potassium carbonate or anhydrous calcium sulfate, filter and then distil, taking precautions to exclude moisture.

g. Methyl ethyl ketone

Dry the commercial product with anhydrous potassium carbonate or anhydrous calcium sulfate, filter and then distil collecting the fraction boiling between 79-80°C. Purify this further through the bisulfite addition compound. Shake methyl ethyl ketone with excess of saturated sodium bisulfite solution until reaction ceases, cool to 0°C. Filter off the bisulfite compound, wash with little ether and dry in the air. Decompose the dry bisulfite compound with a slight excess of sodium carbonate solution and distil in steam. Salt out the ketone from the distillate with potassium carbonate, separate, dry with anhydrous potassium carbonate, and after filtration allow to stand for several hours over anhydrous calcium sulfate, followed by distillation.

It can also be purified by sodium iodide method. Saturate the ketone with sodium iodide by boiling under reflux, filter through a hot water funnel, cool in a freezing mixture, filter off the white crystals and distil (boiling point 79.5°C/760 mm).

h. Benzene

Treat with anhydrous calcium chloride, filter and then place over sodium wire (See Absolute diethyl ether).

Thiophene C_4H_4S with a boiling point of 84°C in benzene is detected as follows. Shake 3 ml of benzene with a solution of 10 mg of isatin in 10 ml concentrated H_2SO_4 and allow the mixture to stand for a short time. A bluish green colour can be observed in the presence of thiophene.

Thiophene can be removed from benzene by shaking with concentrated H_2SO_4 Shake benzene repeatedly with 15% of its volume of concentrated H_2SO_4 in a stoppered separating funnel until acid layer is colourless After each shaking remove the lower layer of acid. Then shake the benzene twice with water to remove acid once with 10% sodium carbonate solution and again with water and finally dry with anhydrous calcium chloride. Distil the benzene after filtration at 80–81°C. If required perfectly dry, introduce sodium wire into the distilled liquid.

i. Petroleum ether

The fractions of refined petroleum commonly used have boiling point 40–60°C, 60–80°C, 80–100°C and 100–120°C. Remove most of the unsaturated aromatic hydrocarbons by shaking two or three times with 10% of the volume of concentrated sulphuric acid, followed with successive portion of concentrated solution of $KMnO_4$ in 10% H_2SO_4 until the colour of the permanganate remains unchanged. Wash the solvent with water, dry over anhydrous calcium chloride and distil. Petroleum ether fractions free from aromatic hydrocarbons are marketted.

j. Ethyl acetate

Reflux a mixture of 1 l of commercial ethyl acetate, 100 ml acetic anhydride and 10 drops of concentrated sulphuric acid for 4 hr and distil. Shake the distillate with 20-30 g potassium carbonate filter and redistil (77°C/760 mm).

k. Pyridine

Reflux over potassium or sodium hydroxide pellets and distil over, excluding moisture (115·5°C / 760 mm).

l. Methylene chloride

Purify the commercial substance by washing with 5% sodium carbonate solution followed by

water, dry over anhydrous calcium chloride and then distil (40–41°C).

m. Chloroform

The commercial product contains 1% alcohol added as stabiliser. It is removed by either of the following procedures.

(i) Shake chloroform two or three times with a small volume of concentrated sulfuric acid followed with water. Dry over anhydrous calcium chloride and distil.

(ii) Shake chloroform five or six times with about half its volume of water, dry over anhydrous calcium chloride for 24 hr and distil (61°C/760 mm). Keep the solvent in dark in order to avoid the photochemical formation of phosgene. It must not be dried with sodium as an explosion may occur.

Reference :

A Text Book of Practical Organic Chemistry. A. I. Vogel (Ed.), Longmans, Green and Co. 3rd Edn. p. 163 (1956).

15. GLASSWARE WASHING

Glassware washing forms a part of general cleanliness required to be maintained in any research laboratory. The procedure to be described has been found satisfactory over several years in a laboratory dealing with variety of biological materials.

As a rule never allow any biological material to dry in any of the glassware. This, if followed rigorously will automatically improve the quality of glassware washing.

Contents of the test tubes in which colour has been developed should never be left for prolonged periods after the readings have been taken. Many of the colour complexes, either in the highly acidic or basic pH, are likely to interact with the glass. Discard these contents immediatley after the experiment and rinse the tubes with tap water and then leave for washing.

Washing :

Rinse with adequate tap water immediately after use. Transfer to detergent—teepol, a commercially available liquid detergent diluted 1 : 10 with tap water. After 2 to 3 hr remove from the detergent. Clean using brush wherever necessary and wash with several rinses of tap water. Transfer to 1 : 10 diluted nitric acid (commercial). Remove after few hours, wash with adequate quantities of water, rinse with distilled and then with glass distilled water. Pipettes should be washed individually.

All the glassware thus washed could be transferred to oven for drying except the pipettes. As a rule pipettes or any graduated glassware should not be oven dried as this is likely to cause distortion of graduations.

16. SAFETY IN THE LABORATORY

General precautions :

1. Care and cleanliness must be practiced by all the laboratory personnel at all times.

2. Smoking is not desirable in a laboratory.
3. Care should be taken to ensure that all gas taps work efficiently and that no gas escapes when the taps are turned off. Do not attempt to find gas leaks by using a lighted match.
4. Electric switches and connections should be repeatedly inspected and kept free from corrosion, so that no danger shall arise from short circuiting or due to the exposure of naked wire from which the insulation has been broken or corroded away.
5. When a laboratory is to be left unattended for any length of time all gas and water taps should be turned off.
6. Proper disposal of waste from a laboratory is a problem of considerable importance. There should be an ample supply of bins of suitable size for disposal of solid materials such as broken glass, porcelain, filter papers, match sticks etc. Lighted matches or smouldering materials should never be thrown into these bins. The bins should be cleared often, atleast daily.
7. Paper should never be used to carry light from one burner to another. Insoluble waste should not be thrown into the sink.
8. If corrosive, poisonous, or radioactive liquids are being thrown down the sink, they should be accompanied by a generous supply of water to ensure that by the time they reach the main drain they will be too dilute to be dangerous.
9. When working in a laboratory it is better to wear some sort of protective coat, apron or overall.
10. All bottles and containers should be carefully and distinctly labelled so that no confusion or possible error can arise when several containers are being filled with different chemicals.
11. Poisonous and dangerous chemicals should be stored separately and in a locked cupboard. Explosive chemicals are kept in a lead lined rectangular box fitted with a good lock, outside the building but close to the lab and readily accessible to the attandant but not to the public (Br_2, PCl_5 and PCl_3 etc.)
12. Greatest care should be taken when handling radioactive materials. Special precautions are given under radioisotope methodology.
13. Many accidents occur in transporting bulky containers from room to room or from one part of the laboratory to another. Bottles should never be carried by the neck. Trays or polythene buckets or suitable trolley should be used.
14. Adequate and conveniently accessible storage of both apparatus and chemicals are essential to the laboratory safety. Bottles containing liquids will be kept on the lower shelves whilst solids are kept on the higher ones.
15. The floor space should be kept perfectly free from spillage, broken glass, straw, paper etc. as their presence may lead to accidents.
16. When dealing with volatile inflammable liquids no flames should be allowed in the vicinity and care should be taken to avoid inhalation of vapours.
17. Cylinders of gases under pressure are best kept upright in a suitable stand well away from any source of heat.
18. When pouring liquids from one container into another keep both vessels well away from the body so that any spillage shall not fall upon the person. When corrosive chemicals are involved it is a good plan to carry out the operation over a sink so that any spillage can be easily flushed away.
19. It is often an advantage to use a glass rod to direct the liquid stream when pouring from one bottle to another.

20. When diluting concentrated sulphuric acid always add the acid to the water kept in ice and not vice versa; add the acid slowly, preferably down a glass rod with stirring to reduce the violence of the reaction.
21. When NaOH is dissolved in water great heat is evolved. Use cold water and add NaOH to it little by little. Do not handle NaOH with unprotected hands.
22. When corrosive, poisonous, volatile and offensive chemicals are being used carry out the operations in a fume hood.
23. Ether or any other volatile inflammable solvents should not be heated directly over a naked flame. Use water baths or heating mantles.
24. When pushing a glass tube or rod through a hole in rubber cork lubricate well with water.
25. Poisonous, radioactive and offensive reagents should never be pipetted by mouth, use propipettes.
26. It is not advisable to use glass stoppers in bottles containing KOH and NaOH. The stoppers are apt to stick and their removal involve breaking the neck of the bottle. Use rubber stoppers or store them in polythene containers.
27. Workers in a biological laboratory are in frequent contact with microbes. Whenever pathogenic microorganisms are handled the work should be done in an area having controlled ventillation and safety cabinets. The persons should have had prior vaccination.
28. There should always be available in easily accessible places fire buckets, containing sand and water. Fire extinguishers should be kept at readily accessible points. They should be sent for filling immediately after being used.
29. In case of emergency, medical attention should be quickly obtained. Meanwhile first aid should be given to the person. A first aid cabinet should be readily accessible and be placed in the charge of a staff member capable of dealing with an emergency. One should learn where to find the following even in the dark; cold water, telephones, fire alarm, exits, first aid box and fire extinguishers. In an emergency, a fire blanket, antidotes, chart about poison antidotes, chart explaining artificial respiration and emergency battery operated lamp etc. should be available.
30. Many accidents are caused by failure to seek advice or, information. Never attempt to use equipment that you do not fully understand.

17. FIRST AID IN ACCIDENTS AND POISONING

Keep the following supplies readily available and in satisfactory condition for use by trained personnel and physicians :

Acids : Acetic acid 20% or vinegar diluted 2 parts water to 1 part vinegar, boric acid 1% solution, tannic acid 4% solution, tannic acid powder.

Alkalies : Aluminium hydroxide gel or tablets, milk of magnesia, sodium bicarbonate, lime water.

Amyl nitrite : 0.3 ml pearls.

Apomorphine hydrochloride : 0.10 mg tablets for hypodermic use.

Atropine sulfate : 0.6 mg tablets for hypodermic use.

Calcium disodium edetate (calcium disodium ethylenediamine tetraacetate) ampoules.

Charcoal : Animal or activated.
Dimercaprol : Ampoules containing 10% solution in oil.
Epinephrine—1 : 1000 Aqueous solution in ampoules.
Magnesium sulfate (Epsom salt).
Morphine sulfate : Solution in ampoules for hypodermic use.
Mustard : Dry powder.
Pentothal sodium ampoules.
Petroleum, lipuid.
Potassium permanganate : 0.2 g Tablets.
Saline: Sterile, normal physiological, in ampoules.
Sodium chloride (common table salt).
Sodium nitrite : 3% Solution in ampoules for IV use.
Sodium thiosulfate : 25% solution in ampoules for iV use.
Sodium thiosulfate powder.

Universal antidote powder :

Mix 2 parts of activated charcoal (burnt toast), 1 part magnesium oxide (milk of magnesia) and 1 part tannic acid (strong tea).

Sterile water in ampoules; Mouth gag and stomach tube; Syringes : 2 ml, 10 ml 50 ml, and needles-26 gauge, ½ inch; 21 gauge, 1½ inches; Oxygen and oxygen mask and respirators (gas masks), self-contained.

General procedure :

General procedure is to be followed in all cases except where deviations are noted under individual poisons.

Remove noxious agent from contact with patient. Place any unconscious or near unconsicous patient in prone position (ie. lying on abdomen) with head turned to one side and tongue pulled forward. Keep patient warm and recumbent. Be prepared to administer mouth to mouth artificial respiration at first suspicion of difficulty in breathing. Do not leave victim unattended. Do not give alcohol except on medical advice. Alcohol increases absorption of some poison. Summon and obtain medical attention as soon as possible, but do not interrupt the above procedures.

If poison is taken by mouth :

Give 2 to 4 glasses of water or milk immediately. Never give anything by mouth to an unconscious person. Induce vomiting immediately. Have patient place his index finger far back on tongue and stroke from side to side (execute this manoeuvre for him if necessary) or give salt water (one tablespoon of salt per glass). Do not induce vomiting if patient has swallowed kerosene, gasoline, strong acid or alkali, or if patient is unconscious. Repeat above procedure until vomited fluid is clear. While administering first aid, try to identify the poison and administer the appropriate antidote. If you cannot identify the poison, give approximately 15 g of 'Universal Antidote' in half a glass of warm water. Save sample of vomitus if possible.

If poison is inhaled :

Try to identify the the poisonous vapour. If it is chlorine, hydrogen sulfide, hydrogen cyanide, phosgene or other highly toxic gas the appropriate type of gas mask should be worn during the exposed portion of the rescue effort. If a suitable mask is not available, rescuers must hold breath as long as they are in contact with poisonous vapours. Do not enter tank of confined space without oxygen

supply and safety ropes attended by assistants. Remove patient to fresh air immediately. At first sign of difficulty in breathing start mouth to mouth artificial respiration, oxygen should be administered only by trained personnel. Continue mouth to mouth artificial respiration until advised by physician to stop.

If poison is in contact with skin :

Identify poison, if possible, and avoid contact. Flood contaminated area of body surface with water for atleast 15 min. Remove all contaminated clothing, including shoes, watches, rings and any other pieces of jewellery. Do not use oils, fats (butter) or soda bicarbonate paste on skin unless these are specifically indicated or you are so instructed by a physician.

If poison is in contact with eyes :

Immediately irrigate both eyes with copious quantities of water (slightly warm, if available). Be sure that eyelids are kept open. If necessary, hold them apart by grasping eye lashes and pulling lids away from eyeball so that wash water gets under both lids. Continue for atleast 15 minutes.

Artificial respiration :

It can be applied with or without adjunct equipment. The use of an S-shaped airway may give increased effectiveness in some situations. The technique is as follows:

Place victim flat on his back. Hold jaw forward. Remove any dentures. See that tongue is forward and there are no obstructions. Hold head backward. Pinch nose closed with thumb and index finger. Blow into mouth until chest rises and resistance of lungs is felt. Break mouth contact and allow victim's lungs to deflate. Repeat cycle about 12 times per min.

Acids, corrosive (acetic, hydrochloric, lactic, nitric, phosphoric, sulfuric etc.) :

External : Use general procedure; Internal : Do not induce vomiting. Do not give carbonates or baking soda (sodium bicarbonate). Give aluminium hydroxide gel or milk of magnesia in large amounts, followed with milk or egg white beaten with water.

Alkalies, caustic (ammonia water, ammonium hydroxide, calcium oxide, caustic potash, caustic soda, lye, quick lime, soda, soda ash) :

External : Use general procedure; Internal ; Do not induce vomiting. Give large amounts of diluted acetic acid (1%), vinegar (1 : 4), citric acid (1%) or lemon juice, followed with milk or egg white beaten with water.

Cyanides (prussic acid, bitter almond oil, cherry laurel water, sodium and potassium cyanide and other cyanides, some fumigating agents) :

Danger : Extremely hazardous gas - wear an approved mask. Speed in first aid and in starting skilled medical treatment is of critical importance. Get medical attention at once.

Inhalation and skin contact : Remove to fresh air immediately. Remove contaminated clothing but keep patient warm. Start and continue mouth to mouth artificial respiration at first sign of difficulty in breathing.

Break amyl nitrite pearl in handkerchief and hold lightly under nose for 15 seconds ; repeat 5 times at about 15 seconds intervals.

Internal : If patient is conscious or when consciousness returns, induce vomiting by giving warm salt water (1 tablespoonful salt per glass of water)

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SECTION-C

APPENDIX

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1. BOILING POINTS

The following boiling points are correct to the nearest degree centigrade.

Substance	°C	Substance	°C
Acetone ..	56	Chloroform ..	62
Acetic acid ..	118	Ether ..	34
Amyl alcohol ..	130	Ethyl alcohol ..	78
Benzene ..	80	Hexane ..	68
Butyl alcohol ..	118	Methyl alcohol ..	65
Caprylic alcohol ..	180	Toluene ..	111
Carbon disulphide ..	46	Water ..	100
		Xylenes ..	138-144

2. DETAILS ON COMMON ACIDS AND ALKALIES AS PURCHASED

Substance	Specific gravity (at room temperature)	Percent by weight	Normality
Ammonium hydroxide (stronger)	0.90	28.0	15.1
Sodium hydroxide (saturated)	1.50	50.0	19.0
Sulfuric acid (concentrated)	1.84	96.0	35.9
Nitric acid (concentrated)	1.42	69.5	15.6
Hydrochloric acid (concentrated)	1.19	36.0	11.7
Phosphoric acid (syrupy)	1.71	85.0	45.0
Acetic acid (glacial)	1.06	99.8	17.6
Perchloric acid	1.54	60.0	9.2

3. EQUIVALENT WEIGHTS OF SOME COMMON CHEMICALS

Name	Formula	Mol. Wt.	Fraction of Mol. Wt.	Eq. Wt.
Hydrochloric acid	HCl	36.46	1.0	36.46
Sulphuric acid	H_2SO_4	98.00	0.5	49.00
Oxalic acid (hydrated)	$(COOH)_2.2H_2O$	126.03	0.5	63.03
Sodium hydroxide	NaOH	40.00	1.0	40.00
Potassium hydroxide	KOH	56.10	1.0	56.10
Sodium carbonate (anhydrous)	Na_2CO_3	106.00	0.5	53.00
Borax	$Na_2B_4O_7.10H_2O$	381.44	0.5	190.72

Potassium permanganate	$KMnO_4$	158.00	0.2	31.60
Potassium dichromate	$K_2Cr_2O_7$	294.20	0.17	49.03
Mohr's salt (Ferrous ammonium sulphate)	$FeSO_4 (NH_4)_2 SO_4.6H_2O$	392.10	1.0	392.10
Ferrous sulphate (crystals)	$FeSO_4.7 H_2O$	278.00	1.0	278.00
Sodium oxalate	$(COONa)_2$	134.00	0.5	67.00
Ammonium oxalate	$(COONH_4)_2.H_2O$	144.08	0.5	72.04
Iodine	I_2	253.80	0.5	126.90
Hypo (Sodium thiosulphate)	$Na_2S_2O_3.5H_2O$	248.20	1.0	248.20

4. PREPARATION OF COMMON BUFFERS

The buffers described in this section are suitable for use either in enzymatic or histochemical studies. The accuracy of the tables is within $\pm$0.05 pH at 23°C. In most cases the pH values will not be off by more than $\pm$0.12 pH even at 37°C and at molarities slightly different from those given (usually 0.05 M).

a. Glycine-HCl Buffer pH 2.2-3.6

Stock solutions: A- 0.2 M Solution of glycine (15.01 g in 1 l); B - 0.2 M HCl.

50 ml of A + X ml of B, diluted to a total of 200 ml.

X	pH	X	pH
5.0	3.6	16.8	2.8
6.4	3.4	24.2	2.6
8.2	3.2	32.4	2.4
11.4	3.0	44.0	2.2

b. Citrate-Phosphate Buffer pH 2.6-7.0

Stock solutions: A-0.1 M Solution of citric acid (19.21 g in 1 l); B-0.2 M Solution of dibasic sodium phosphate (53.65 g of $Na_2 HPO_4. 7H_2O$ or 71.7 g of $Na_2 HPO_4.12H_2O$ in 1 l).

X ml of A + Y ml of B, diluted to a total of 100 ml.

X	Y	pH	X	Y	pH
44.6	5.4	2.6	24.3	25.7	5.0
42.2	7.8	2.8	23.3	26.7	5.2
39.8	10.2	3.0	22.2	27.8	5.4
37.7	12.3	3.2	21.0	29.0	5.6
35.9	14.1	3.4	19.7	30.3	5.8
33.9	16.1	3.6	17.9	32.1	6.0
32.3	17.7	3.8	16.9	33.1	6.2
30.7	19.3	4.0	15.4	34.6	6.4
29.4	20.6	4.2	13.6	36.4	6.6
27.8	22.2	4.4	9.1	40.9	6.8
26.7	23.3	4.6	6.5	43.5	7.0
25.2	24.8	4.8			

c. Citrate Buffer pH 3.0 - 6.2

Stock solutions : A-0.1 M Solution of citric acid (21.01 g in 1 l) ; B-0.1 M Solution of sodium citrate (29.41 g $C_6H_5O_7$ Na_3.2H O in 1 l, the use of the salt with 5.5 H_2O is not recommended).

X ml of A + Y ml of B, diluted to a total of 100 ml.

X	Y	pH	X	Y	pH
46.5	3.5	3.0	23.0	27.0	4.8
43.7	6.3	3.2	20.5	29.5	5.0
40.0	10.0	3.4	18.0	32.0	5.2
37.0	13.0	3.6	16.0	34.0	5.4
35.0	15.0	3.8	13.7	36.3	5.6
33.0	17.0	4.0	11.8	38.2	5.8
31.5	18.5	4.2	9.5	40.5	6.0
28.0	22.0	4.4	7.2	42.8	6.2
25.5	24.5	4.6			

d. Acetate Buffer pH 3.6 - 5.6

Stock solutions : A-0.2 M Solution of acetic acid (11.55 ml in 1 l) ; B-0.2 M Solution of sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na$. $3H_2O$ in 1 l).

X ml of A + Y ml of B, diluted to a total of 100 ml.

X	Y	pH	X	Y	pH
46.3	3.7	3 6	20.0	30.0	4.8
44.0	6.0	3 8	14.8	35.2	5.0
41.0	9.0	4.0	10.5	39.5	5.2
36.8	13.2	4.2	8.8	41.2	5.4
30.5	19.5	4.4	4.8	45.2	5.6
25.5	24.5	4.6			

e. Succinate Buffer pH 3.8 - 6.0

Stock solutions : A-0.2 M Solution of succinic acid (23.6 g in 1 l) ; B-0.2 M NaOH.

25 ml of A + Y ml of B, diluted to a total of 100 ml.

Y	pH	Y	pH	Y	pH
7.5	3.8	20.0	4.6	34.2	5.4
10.0	4.0	23.5	4.8	37.5	5.6
13.3	4.2	26.7	5.0	40.7	5.8
16.7	4.4	30.3	5.2	43.5	6.0

f. Phspohate Buffer pH 5.7 - 8.0

Stock solutions : A-$Na_2HPO_4.2H_2O$, 0.2 M Solution contains 35.61 g/l or $Na_2HPO_4.12H_2O$, 0.2 M solution contains 71.64 g/l ; B-$NaH_2PO_4.H_2O$, 0.2 M Solution contains 27.67 g/l or $NaH_2PO_4.2H_2O$, 0.2 M solution contains 31.21 g/l

X ml of A + Y ml of B, diluted to a total of 200 ml.

X	Y	pH	X	Y	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

g. Phosphate Buffer pH 5.8 - 8.0

50 of ml 0.1 M KH_2PO_4 (13.60 g/l), Y ml 0.1 M NaOH, diluted to 100 ml.

Y	pH	Y	pH	Y	pH
3.6	5.8	16.4	6.6	39.1	7.4
4.6	5.9	19.3	6.7	40.9	7.5
5.6	6.0	22.4	6.8	42.4	7.6
6.8	6.1	25.9	6.9	43.5	7.7
8.1	6.2	29.1	7.0	44.5	7.8
9.7	6.3	32.1	7.1	45.3	7.9
11.6	6.4	34.7	7.2	46.1	8.0
13.9	6.5	37.0	7.3		

h. Sodium bicarbonate-5% CO_2 Buffer solutions pH 6.0 - 8.0

Calculated from the Henderson-Hasselbalch equation, $pH = pK + \log \frac{(HCO_3)}{(CO_2)}$, concentrations of bicarbonate are approximately correct when atmospheric pressure varies between 725 and 760 mm Hg. For temperatures below 37°C (down to 20°C) the bicarbonate concentration given in the table should be decreased by approximately 1.88% per degree to give the required pH.

pH at 37°C	M conc. of $NaHCO_3$ in equilibrium with gas phase containing 5% CO_2	pH at 37°C	M conc. $NaHCO_3$ in equilibrium with gas phase containing 5% CO_2
6.0	5.86×10^{-4}	7.2	9.29×10^{-3}
6.2	9.29×10^{-4}	7.4	1.47×10^{-2}
6.4	1.47×10^{-3}	7.6	2.33×10^{-2}
6.6	2.33×10^{-3}	7.8	3.70×10^{-2}
6.8	3.70×10^{-3}	8.0	5.86×10^{-2}
7.0	5.86×10^{-3}		

i. Barbital Buffer pH 6.8 - 9.2

Stock soultions : A - 0.2 M Solution of sodium barbital (41.2 g in 1 l); B - 0.2 M HCl.

50 ml of A + Y ml of B, diluted to a total of 200 ml.

Y	pH	Y	pH	Y	pH
1.5	9.2	12.7	8.2	32.5	7.4
2.5	9.0	17.5	8.0	39.0	7.2
4.0	8.8	22.5	7.8	43.0	7.0
6.0	8.6	27.5	7.6	45.0	6.8
9.0	8.4				

Note :

Solutions more concentrated than 0.05 M may crystallise on standing, especially in the cold.

j. Tri - HCl Buffer pH 7.2 - 9.0

Stock solutions : A - 0.2 M Solution of tris - (hydroxymethyl) aminomethane (24.2 g in 1 l) ; B - 0.2 M HCl.

50 ml of A + Y ml of B, diluted to a total of 200 ml.

Y	pH	Y	pH	Y	pH
5.0	9.0	21.9	8.2	38.4	7.6
8.1	8.8	26.8	8.0	41.4	7.4
12.2	8.6	32.5	7.8	44.2	7.2
16.5	8.4				

k. Krebs-Ringer Phosphate and Bicarbonate solutions pH 7.4

Solutions :

1. 0.90% NaCl — 0.154 M
2. 1.15% KCl — 0.154 M
3. 1.22% $CaCl_2$ — 0.11 M (5 ml equivalent to 11 ml 0.1 N $AgNO_3$)

4. 2.11% KH_2PO_4 — 0.154 M
5. 3.82% $MgSO_4.7H_2O$ — 0.154 M
6. 1.30% $NaHCO_3$ — 0.154 M (gas with CO_2 for 1 hr)
7. Phosphate buffer pH 7.4 — 0.1 M (17.8 g $Na_2HPO_4.2H_2O$ + 20 ml 1 N HCl; dilute to 1 l).

To prepare the Krebs Ringer solution, the following amounts of the above are mixed : 100 parts of solution 1, 4 parts of solution 2, 3 parts of solution 3, 1 part of solution 4 and 1 part of solution 5.

Note :

All solutions are isotonic with rat serum, hence can be mixed in any proportion yielding mixtures (of differing composition) which are still isotonic.

If Krebs-Ringer bicarbonate is desired, 21 parts of solution 6 are added. The solution is then gassed for 10 min with 50% CO_2. For aerobic experiments 5% CO_2-95% O_2 mixture is usually used and for anaerobic experiments, 5% CO_2-95% N_2. After mixing and gassing the solution, it should be kept in a glass-stoppered vessel in the cold, until ready for use. The manometric flasks containing the solution should be attached to the manometers and gassed as soon as possible.

If Krebs-Ringer phosphate is desired, omit solution 4 from the Krebs-Ringer solution and add 20 parts of solution 7. This solution after mixing is gassed with either O_2, N_2 or air, depending on the gas phase desired. A precipitate of calcium phosphate forms which is suspended by shaking before use.

To simplify the preparation and handling of the above solutions, it has been found convenient to make up solutions number 1 to 5 in concentrations five times those listed. The more concentrated solutions are stable for months when stored in the cold. To make a stock Krebs-Ringer solution, solutions 1 to 5 are made up in the proportions indicated above (taking into account the fact that these stock solutions are five times the concentration required). This stock Krebs-Ringer solution will keep well in the cold for about one week. To make up the Krebs-Ringer bicarbonate solution, 16 ml of solution 6 are diluted to 100 ml with the stock Krebs-Ringer solution. To make up the Krebs-Ringer phosphate, 10 ml of solution 7 are diluted to 100 ml with the stock Krebs-Ringer solution.

An alternative medium buffered with phosphate is found to be particularly suitable for kidney slices. Except for the absence of bicarbonate the medium resembled mammalian extraclelualr fluid. The medium has the following composition :

Constituent	Concentration (M)	Parts mixed for whole medium (ml)
NaCl	0.154	232
KCl	0.154	8
$MgSO_4$	0.154	2
$CaCl_2$	0.110	6

The mixture is adjusted to pH 7.4 by the addition of 0.1N NaOH. To this add 12 ml of M/15 phosphate buffer, pH 7.4. (This is prepared by mixing 80.8 ml of M/15 Na_2HPO_4 with 19.2 ml of M/15 KH_2PO_4). To avoid precipitation, the calcium solution and phosphate buffer are not brought together in less than the final volume. The completed solution, however, is found to be stable.

Glucose is added to this medium in the amount of 0.4 ml of 5% solution to 19.6 ml of medium. The resulting medium contains in millimoles per liter approximately the following : Na^+, 140; K^+, 5 ; Ca^{++}, 2.5; Mg^{++}, 1; Cl^-, 144; PO_4^{---}, 3 and SO_4^{--} = 1, giving a total ionic strength of 0 158.

l. Borate Buffer pH 8.1 - 9.0

50 ml of 0.025 M $Na_2B_4O_7.10H_2O$ (9.525 g/l) + Y ml 0.1 M HCl, diluted to 100 ml.

Y	pH	Y	pH	Y	pH
19.7	8.1	15.2	8.5	9.4	8.8
18.8	8.2	13.5	8.6	7.1	8.9
17.7	8.3	11.6	8.7	4.6	9.0
16.8	8.4				

m. Glycine - NaOH Buffer pH 8.6 - 10.6

25 ml of 0.2 M Glycine (15.01 g/l) + Y ml 0.2 N NaOH, diluted to 100 ml.

$$\frac{dpH}{dt} = -0.026 \text{ pH units/degree}$$

Y	pH	Y	pH	Y	pH
2.0	8.6	8.4	9.4	16.0	10.0
3.0	8.8	11.2	9.6	19.3	10.4
4.4	9.0	13.6	9.8	22.75	10.6
6.0	9.2				

n. Sodium Carbonate Bicarbonate Buffer pH 9.2 - 10 8

$Na_2CO_3.10H_2O$, 0.1 M Solution contains 28.62 g/l; $NaHCO_3$, 0.1 M Solution contains 8.40 g/l.

X ml 0.1 M Na_2CO_3 and Y ml 0.1 M $NaHCO_3$ are mixed.

		pH	
X	Y	20°C	37°C
10	90	9.2	8.8
20	80	9.4	9.1
30	70	9.5	9.4
40	60	9.8	9.5
50	50	9.9	9.7
60	40	10.1	9.9
70	30	10.3	10.1
80	20	10.5	10.3
90	10	10.8	10.6

o. Borate Buffer pH 9.3 - 10.7

50 ml 0.025 M $Na_2B_4O_7.10H_2O$ (9.525 g/l) + Y ml 0.1 M NaOH, diluted to 100 ml

Y	pH	Y	pH	Y	pH
3.6	9.3	8.8	9.5	13.1	9.7
6.2	9.4	11.1	9.6	15.0	9.8

16.7	9.9	20.5	10.2	22.7	10.5
18.3	10.0	12.3	10.3	23.3	10.6
19.5	10.1	22.1	10.4	23.8	10.7

p. Carbonate Buffer pH 9.7 – 10.9

50 ml of 0.05 M $NaHCO_3$ (4.20 g/l) + Y ml 0.1 M NaOH, diluted to 100 ml.

Y	pH	Y	pH	Y	pH
6.2	9.7	13.8	10.2	19.1	10.6
7.6	9.8	15.2	10.3	20.2	10.7
9.1	9.9	16.5	10.4	21.2	10.8
10.7	10.0	17.8	10.5	22.0	10.9
12.2	10.1				

References:

1. Methods in Enzymology. S.P. Colowick and N.O. Kaplan (Eds.) Vol. I, p. 138 (1955).
2. Data for Biochemical Research, R.M.C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones (Eds.) Oxford University Press, 2nd Edn. p. 483 (1974).

5. GEL FILTRATION MEDIA

Type	Approx. exclusion limit M. Wt. x 1000	Fractionation range M. Wt x 1000	Water regain (g water/g dry gel)	Bed volume (ml/g dry gel) *	Particle size (μm)
Sephadex **					
G-10	0.7	up to 0.7	1.0 ± 0.1	2-3	40-120
G-15	1.5	up to 1.5	1.5 ± 0.2	2.5-3.5	40-120
G-25	5.0	0.1-5.0	2.5 ± 0.2	5	
Fine					20-80
Coarse					100-300
G-50	10.0	0.5-10.0	5.0 ± 0.3	10	
Fine					20-80
Coarse					100-300
G-75	50.0	1.0-50	7.5 ± 0.5	12-15	40-120
G-100	100.0	5.0-100.0	10.0 ± 1.0	15-20	40-120
G-150	150.0	5.0-150.0	15.0 ± 1.5	20-30	40-120
G-200	200.0	5.0-200.0	20.0 ± 2.0	30-40	40-120
LH 20***		0.1-4.0 in ethanol	2.1 (H_2O)	4 (H_2O)	25-100
		0.1-2.0 in $CHCl_3$	1.8 ($CHCl_3$, ethanol)	3-3.5 ($CHCl_3$), ethanol,	

Bio-gel					
P-2	1.6	0.2-2.0	1.5	3.8	Range 50-
P-4	3.6	0.5-4.0	2.4	5.8	100, 100-
P-6	4.6	1.0-5.0	3.7	8.8	200, available
P-10	10.0	5.0-17.0	4.5	12.4	in all; 200-
P-30	30.0	20.0-50.0	5.7	14.8	400 in P-2,P-4
P-60	60.0	30.0-70.0	7.2	19.0	P-6, P-10.
P-100	100.0	40.0-100.0	7.5	19.0	
P-150	150.0	50.0-150.0	9.2	24.0	
P-200	200.0	80.0-300.0	14.7	34.0	
P-300	300.0	100.0-400.0	18.0	40.0	

* Volume of 1 g dry material when in equilibrium with an excess of water.

** Data refer to the bead form of Sephadex and not the original crushed powders.

*** Produced by alkylation of Sephadex G-25 and can be used to fractionate substances dissolved in organic solvents.

6. ION EXCHANGE RESINS

a. Cation Exchange Resins

Designation	Functional group	Cross linkage (% divinyl-benzene)	Designation	Functional group	Cross linkage (% divinyl benzene)
Dowex 50-X (AG50W-X1) *50-100	ϕ SO_3	1	Zeo Karb 225	$\phi - SO_3$	1-20
			IR-120 CP	,,	1-12
Dowex 50-X2 (AG50W-X2) *50-400	,,	2	CG 120 AR *100-600	,,	8
			Bio-Rex 70	Acrylic acid $R_1 - COO^-$	10
Dowex 50-X4 (AG 50 W-X4) *20-400	,,	4	Bio-Rex 62	ϕ POO^-	5.5
			Bio-Rex 63	$\phi - POO_2^-$	5.5
Dowex 50-X5 (AG50W-X5) *minus 400	,,	5	Bio-Rex 40	$\phi - O^-$; $R - CH_2SO_3^-$	—
			Chelex 100	ϕ-N(CH_2 $COO^-)_2$	—
Dowex 50-X8 (AG50W-X8) *20-400	,,	8	Zeo Karb 226	R_2-COO^-	2.5-4.5
			IRC-50 CP	,,	5
Dowex 50-X12 (AG50W-X12) *50-400	,,	12	IRC-50 AR	,,	,,
			CG-50 AR *100-600	,,	,,
Dowex 50-X16 (AG50W-X16) *20-400	,,	16	XE-89 CP	Acrylic acid $R_1 - COO^-$	—

b. Anion Exchange Resins

Dowex 1-X1 (AG1-X1) *50-100	ϕ-CH_2N^+-$(CH_3)_3$	1	IRA-401 AR	ϕ-CH_2N^+-$(CH_3)_3$	4
Dowex 1-X2 (AG1-X2) *50-400	,,	2	IRA-402 CP	,,	6
Dowex 1-X4 (AG1-X4) *20-400	,,	4	IRA-400 CP	,,	8
Dowex 1-X8 (AG1-X8) *20-400	,,	8	IRA-400 AR *100-400	,,	,,
Dowex 1-X10(AG1-X10) *50-400	,,	10	CG-400 AR	,,	8
Dowex 21K (AG-21K)			IRA-410 CP	ϕ-CH_2N^+-$(CH_3)_2$-CH_2OH	8
Dowex 2-X4 (AG2-X4) *20-50	ϕ-CH_2N^+-$(CH_3)_2$-C_2H_4OH	4	Dowex 3-X4 (AG3-X4) *20-400	Polyamine	4
Dowex 2-X8 (AG2-X8) *20-400	,,	8	IR-45 AR	,,	
Dowex 2-X10(AG2-X10) *50-400	,,	10	De-Acidite G	ϕ-CH_2N^+-$(C_2H_5)_2$	2-9
De-Acidite FF	ϕ-CH_2N^+-$(CH_3)_3$	2-9	De-Acidite H	Mixed base tertiary amine and quaternary ammonium.	2-9

* Value denote dry mesh size.

c. Cellulose Ion Exchange Resins

The ion exchange celluloses usually have maximum capacities in the range 0.2–0.9 mEq/g. The definition of parcticle size is somewhat arbitrary because of the rod-like shape of the cellulose particles. Prolonged exposure to solutions more acidic than pH 4 should be avoided, although brief contact with 1 N hydrochloric acid is not harmful.

Designation	Chemical Type	Acidity/ Basicity	Remarks
Cation exchangers			
CM-Cellulose	Carboxymethyl (-O-CH_2CO_2H)	Weak	Useful for the fractionation of neutral and basic proteins.
P-Cellulose	Phosphorylated (-O-PO_3H_2)	Strong and weak	Dibasic; binds basic proteins strongly
SE-Cellulose	Sulphoethyl	Strong	—
Hydroxylapatit Cellulose	Calciumphosphate		Hydroxylapatit in combination with cellulose gives faster flow rates than those obtained with hydroxylapatit alone.

Anion exchangers

DEAE-Cellulose	Diethylaminoethyl -O $C_2H_4N(C_2H_5)_2$	Moderately strong	Widely used for the chromatography of acidic and neutral proteins.
TEAE-Cellulose	Triethylaminoethyl	Strong	Probably contains some strongly basic quarternary amine groups. Absence of carboxylic groups may result in sharper separation.
AE-Cellulose	Aminoethyl - $OC_2H_4NH_3$	Weak	—
ECTEOLA-Cellulose	Mixed amines	Intermediate	Used for the chromatography of nucleic acids.
PAB-Cellulose	Para-aminobenzoyl -O $CH_2C_6H_4NH_2$	Weak	Applicable to the chromatography of high molecular weight nucleic acids. Amino group can be diazotised and coupled with proteins to yield specific absorbents.
GE-Cellulose	Guanidoethyl $-OC_2H_4NHC-(NH_2-NH_2)^+Cl^-$	Strong	—

d. Sephadex and Bio-Gel Ion Exchange Resins

Sephadex ionexchangers are derivatives of sephadex G-25 and G-50. Owing to their porous structure and high degree of substitution, they have a high capacity even for large molecules. Bio-gel exchangers are derivatives of Bio-gel P polyacrylamide gels.

Designation	Functional group	Bed volume (ml/g dry gel)	Designation	Functional group	Bed volume (ml/g dry gel)
Cation exchangers			*Anion exchangers*		
			DEAE-Sephadex A-25	$-O-C_2H_4-N^+H(C_2H_5)_2$	—
CM-Sephadex C-25	$-O-CH_2-COOH$	—			
			DEAE-Sephadex A-50	„	15-20
CM-Sephadex C-50	„	15-20	Bio-gel DE 2	$-COO-C_2H_4-N^+H(C_2H_5)_2$	—
SE-Sephadex C-25	$-O-C_2H_4-SO_3H$	—			
			Bio-gel DM 2	$-CONH-CH_2-N^+H(C_2H_5)_2$	7
SE-Sephadex C-50	„	15-20			
Bio-gel CM 2	-COOH	5.6,5.5	Bio-gel DM 30(1)	„	25-30
Bio-gel CM 30 (1)	„	68,35.5	Bio-gel DM 30(2)	„	20
Bio-gel CM 30 (2)	„	50, 40	Bio-gel DM 100	„	50
Bio-gel CM 100	„	124, 45			

For highest absorption capacity C–25 and A 25 should he used for substances with a molecular weight up to about 10,000 and C–50 and A–50 for larger molecules.

7. AMMONIUM SULPHATE FRACTIONATION OF PROTEINS

The chart shows the amount of solid $(NH_4)_2SO_4$ required to bring a solution of known initial saturation to a desired final saturation. In this case, the saturation is relative to full saturation at 0°C.

	Final concentration of ammonium sulphate % saturation at 0°C																
*	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulphate to add to 100 ml of solution																
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
55								0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
65										0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.2	6.6	10.1	13.7	17.4
80													0	3.3	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7.0
95																0	3.5
100																	0

* Initial concentration of ammonium sulphate, % saturation

Formulae for ammonium sulphate additions: For the addition of saturated $(NH_4)_2SO_4$ solutions, the volume changes on mixing are negligible. The volume (v) of saturated $(NH_4)_2SO_4$ (in ml) which has to be added to 100 ml of solution of initial saturation S_1 to produce a final saturation S_2 is given by the equation

$$V = \frac{100\ (S_2 - S_1)}{1 - S_2}\ .$$

S_1 and S_2 are expressed as fractions of the saturated solution, e.g. $S_1 = 0.5$ and $S_2 = 0.7$.

Saturated ammonium sulphate solution at various temperatures

	Temperature (°C)				
	0	10	20	25	30
Moles $(NH_4)_2SO_4$ per 1000g H_2O	5.35	5.53	5.73	5.82	5.91
Percentage by weight	41.42	42.22	43.09	43.47	43.85
g $(NH_4)_2SO_4$ required to saturate 1000 ml H_2O	706.8	730.5	755.8	766.8	777.5
g $(NH_4)_2SO_4$ per l of saturated solution	514.8	525.2	536.5	541.2	545.9
Molarity of saturated solution	3.90	3.97	4.06	4.10	4.13

Reference:

Data for Biochemical Research, R. M. C. Dawson, D. C. Elliott, W.H. Elliott and K. M. Jones, (Eds.). Oxford University Press, 2nd Edn. (1974).

8. SOLUTIONS USED IN FORMING DENSITY GRADIENTS

a. Sucrose

Conc. of sucrose		Density (g/ml)		Viscosity (centipoises)		
% W/W	g/l of soln. at 20°C	at 0°C	at 20°C	at 0°C	at 5°C	at 20°C
5	50.9	1.020	1.018			1.14
10	103.8	1.041	1.038			1.33
15	158.9	1.063	1.059			1.59
20	216.2	1.085	1.081	3.82	3.17	1.95
30	338.1	1.133	1.127			3.19
35	402.9	1.158	1.151			4.32
40	470.6	1.183	1.176	14.8	11.6	6.17
45	541.3	1.210	1.203			9.38
50	614.8	1.238	1.230			15.4
55	691.9	1.266	1.258			28.1
60	771.9	1.296	1.286	238	155	58.5
65	855.6	1.326	1.316			147

b. Caesium chloride

Conc of CsCl		Density (g/ml)		Conc. of CsCl		Density (g/ml)	
% W/W	g/l of soln. at 20°C	at 0°C	at 20°C	% W/W	g/l of soln. at 20°C	at 0°C	at 20°C
5	51.9	1.040	1.038	40	568.8	1.432	1.422
10	108.0	1.084	1.080	45	675.0	1.511	1.500
15	168.9	1.131	1.126	50	793.0	1.598	1.586
20	235.2	1.181	1.176	55	924.5	1.695	1.681
25	307.7	1.236	1.231	60	1073	1.804	1.789
30	386.4	1.296	1.288	65	1241		1.910
35	473.2	1.361	1.352				

The concentration of caesium chloride solution corresponding to any desired density may be calculated form the formula $W(\%) = 137.48 - 138.11\ (1/pss)$, where pss is the desired density at 25°C.

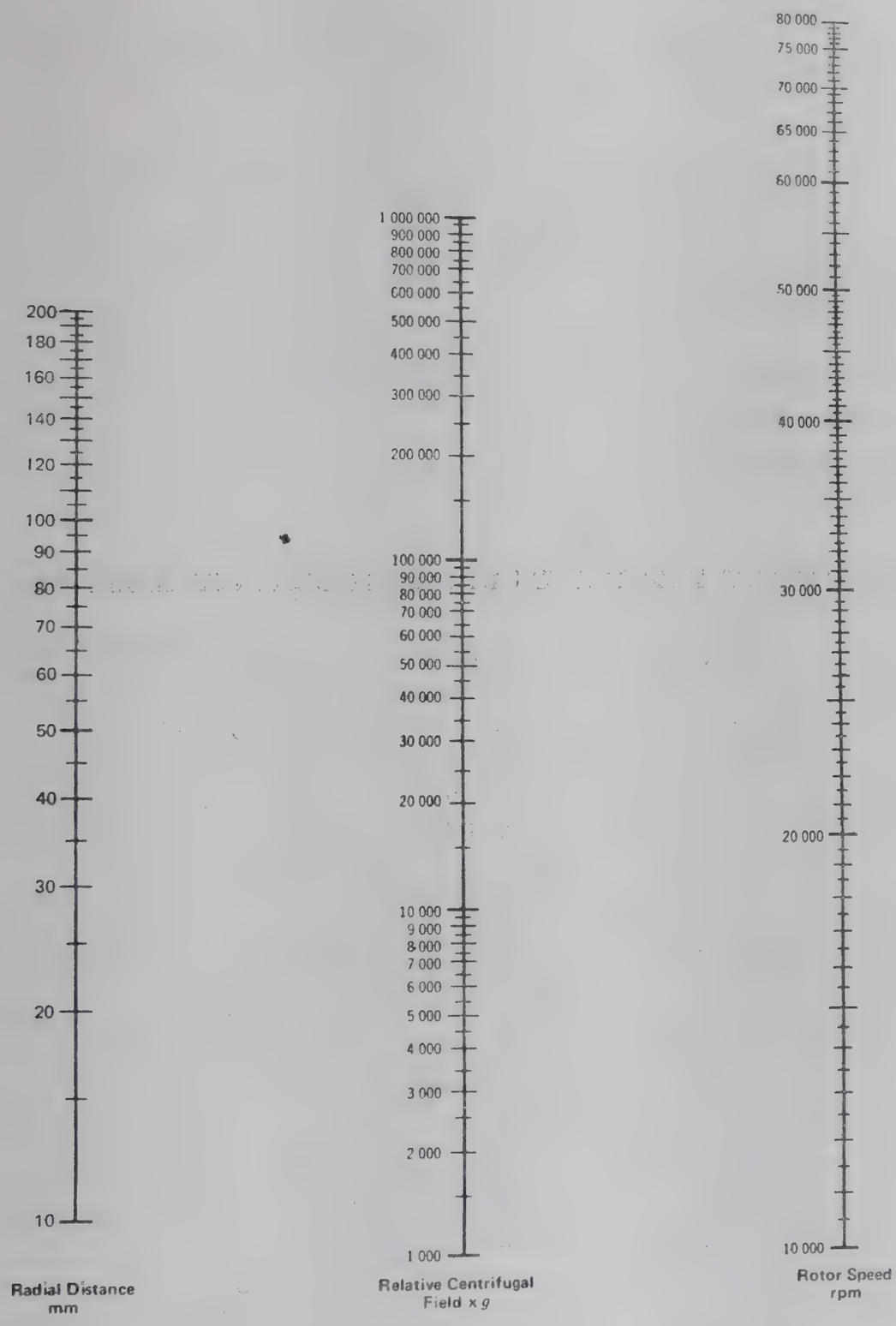
c. Ficoll

Conc. of Ficoll % (W/V)	Density 20°C	Viscosity (centipoises 20°C)
10	1.033	5
20	1.068	20
30	1.103	60
40	1.138	180
50	1.171	600

References :

1. Prog. Biophys. Biophys. Chem. 9. 357 (1959).
2. International Critical Tables, Vol. 2, p. 343 (1927).

9. NOMOGRAM FOR COMPUTING RELATIVE CENTRIFUGAL FORCE



To calculate the RCF value at any point along the tube or bottle being used in the centrifuge, measure the radius in mm from the centre of the centrifuge spindle to the particular point. A straight line connecting the value of the radius on the left of the nomogram with the centrifuge speed on the right will enable the RCF value to be read off on the centre column. For calculation of RCF, Refer Centrifugation in Section A.

10. CLASSIFICATION OF WHATMAN'S FILTER PAPERS

Type of paper	Speed of filtration				
	Fast*	Intermediate	Medium**	Intermediate	Slow***
Ordinary unwashed	4	1	2	3	5
Acid washed	31	43	30		32
	41		40		42
Hardened; acid	54		52		50
washed; tough when	531		530	544	542
wet; will withstand	541		540		
alkalies					

* Retains coarse particles

** Retains medium particles

*** Retains fine particles

11. SOME RADIOACTIVE ISOTOPES USED IN BIOCHEMICAL INVESTIGATION

Element (mass no.)	Radiation	Half-life	Energy of radiation (Me V) practices	γ-rays
^{3}H	β^-	12.1 yr	0.0185	..
^{14}C	β^-	5100 yr	0.156	..
^{22}Na	β^+,γ	2.6 yr	0.58	1.3
^{24}Na	β^-,γ	14.8 hr	1.39	1.38,2.76
^{28}Mg	β^-	21.4 hr	0.459	..
^{32}P	β^-	14.3 days	1.71	..
^{35}S	β^-	87.1 days	0.169	..
^{36}Cl	β^+,k,β^-	2 x 10^6 yr (β^-)	0.64	..
^{42}K	β^-,γ	12 4 hr	2.04 (25%)	1.4,2.1
			3.58 (75%)	
^{45}Ca	β^-	152 days	0.260	..
^{51}Cr	k,γ	28 days		0.323,0.237
^{52}Mn	β^+(35%)	5.8 days	0.58	1.0, 0.73
	k,(65%)			0.94,1.46
^{54}Mn	k,γ	310 days	..	0.835
^{55}Fe	k only	2.94 yr	..	..
^{59}Fe	β^-,γ	46.3 days	0.46 (50%)	1.3 (50%)
			0.26 (50%)	1.1 (50%)
^{57}Co	γ	270 days		0.136 (10%)
				0.122 (88%)
^{58}Co	β^+(14.5%),γ	72 days	0.472	0.805
^{60}Co	β^-,γ	5.3 yr	0.31	1.16,1.32
^{65}Zn	β^+(1.3%)	250 days	0.32	1.14 (46% of k)
	k(98.7%)			

^{86}Rb	β^-,γ	19.5 days	1.822 (80%) 0.716 (20%)	1.081
^{125}I	γ	60 days	..	0.035
^{131}I	β^-,γ	8.1 days	0.605 (86%) 0.25 (14%)	0.637,0.363 0.282,0.08

12. COMPOSITION OF LIQUID SCINTILLATION COUNTING FLUIDS

For counting aqueous samples such as plasma and urine, Bray's mixture is used. The samples in organic phase are counted in toluene scintillator. Tissues are solubilised using the organic base hyamine hydroxide and then counted in toluene scintillator. The scintillator (10 – 20 ml) is pipetted into the specially made counting glass vials. The sample (0.1 – 1 ml) is introduced into the scintillator solution, mixed and the vials placed in the counting chamber for counting.

	Toluene scintillator	Bray's mixture
Primary scintillator - POP (2, 5, Diphenyloxazole)	5g	4 g
Secondary scintillator-POPOP – 1, 4 bis – 2 (5 phenyloxazolyl benzene)	0.3g	0.2g
Solvents		
Toluene	1000 ml	—
Naphthalene	—	60g
Methanol	—	100 ml
Ethylene glycol	—	20 ml
1 - 4 Dioxane	—	Make to 1000 ml with dioxane

13. RELATION BETWEEN TRANSMITTANCE AND OPTICAL DENSITY

Transmittance	O.D	Transmittance	O.D	Transmittance	O.D	Transmittance	O.D
100	0.000	86	0.066	72	0.143	58	0.237
99	0.004	85	0.071	71	0.149	57	0.244
98	0.009	84	0.076	70	0.155	56	0.252
97	0.013	83	0.081	69	0.161	55	0.260
96	0.018	82	0.086	68	0.168	54	0.268
95	0.022	81	0.092	67	0.174	53	0.276
94	0.027	80	0.097	66	0.181	52	0.284
93	0.032	79	0.102	65	0.187	51	0.292
92	0.036	78	0.108	64	0.194	50	0.301
91	0.041	77	0.114	63	0.201	49	0.310
90	0.046	76	0.119	62	0.208	48	0.319
89	0.051	75	0.125	61	0.215	47	0.328
88	0.056	74	0.131	60	0.222	46	0.337
87	0.061	73	0.137	59	0.229	45	0.347

44	0.357	33	0 482	22	0.658	11	0.989
43	0.367	32	0.495	21	0.678	10	1.000
42	0.377	31	0.509	20	0.699	9	1.046
41	0.387	30	0.523	19	0.721	8	1.097
40	0.398	29	0.538	18	0.745	7	1.155
39	0.409	28	0.552	17	0.770	6	1.222
38	0.420	27	0.569	16	0.796	5	1.301
37	0.432	26	0.585	15	0.824	4	1.398
36	0.444	25	0.602	14	0.854	3	1.523
35	0.456	24	0.620	13	0.886	2	1.699
34	0.469	23	0.638	12	0.921	1	2.000

14. HAGEDORN AND JENSEN CONVERSION TABLE FOR BLOOD GLUCOSE

Milliliter 0.005 N $Na_2S_2O_3$ used and mg glucose present

ml	*0.00*	*0.01*	*0.02*	*0.03*	*0.04*	*0.05*	*0.06*	*0.07*	*0.08*	*0.09*
0.0	0.385	0.382	0.379	0.376	0.373	0.370	0.367	0.364	0.361	0.358
0.1	0.355	0.352	0.350	0.348	0.345	0.343	0.341	0.338	0.336	0.333
0.2	0.331	0.329	0.327	0.325	0.323	0.321	0.318	0.316	0.314	0.312
0.3	0.310	0.308	0.306	0.304	0.302	0.300	0.298	0.296	0.294	0.292
0.4	0.290	0.288	0.286	0.284	0.282	0.280	0.278	0.276	0.274	0.272
0.5	0.270	0.268	0.266	0.264	0.262	0.260	0.259	0.257	0.255	0.253
0.6	0.251	0.249	0.247	0.245	0.243	0.241	0.240	0.238	0.236	0.234
0.7	0.232	0.230	0.228	0.226	0.224	0.222	0.221	0.219	0.217	0.215
0.8	0.213	0.211	0.209	0.208	0.206	0.204	0.202	0.200	0.199	0.197
0.9	0.195	0.193	0.191	0.190	0.188	0.186	0.184	0.182	0.181	0.179
1.0	0.177	0.175	0.173	0.172	0.170	0.168	0.166	0.164	0.163	0.161
1.1	0.159	0.157	0.155	0.154	0.152	0.150	0.148	0.146	0.145	0.143
1.2	0.141	0.139	0.138	0.136	0.134	0.132	0.131	0.129	0.127	0.125
1.3	0.124	0.122	0.120	0.119	0.117	0.115	0.113	0.111	0.110	0.108
1.4	0.106	0.104	0.102	0.101	0.099	0.097	0.095	0.093	0.092	0.090
1.5	0.088	0.086	0.084	0.083	0.081	0.079	0.077	0.075	0.074	0.072
1.6	0.070	0.068	0.066	0.065	0.063	0.061	0.059	0.057	0.056	0.054
1.7	0.052	0.050	0.048	0.047	0.045	0.043	0.041	0.039	0.038	0.036
1.8	0.034	0.032	0.031	0.029	0.027	0.025	0.024	0.022	0.020	0.019
1.9	0.017	0.015	0.014	0.012	0.010	0.008	0.007	0.005	0.003	0.002

Reference :

Practical Physiological Chemistry. P. B. Hawk, B. L. Oser and W. H. Summerson (Eds.), McGraw - Hill Book Company, Inc. 13th Edn. p. 577 (1947).

15. INTERNATIONAL SYSTEM OF UNITS AND CONVERSION TABLES

The metric system of units based on the meter (M), kilogram (Kg), second (sec), ampere (A), Kelvin degree (°k), and Candela (Cd), is known as the International System of Units. Also called as MSKA system.

Metric equivalents

1 Kilo	=	2.2 lb
1 lb	=	0.454 Kg
1 oz (avoir)	=	28.35 g
1 litre	=	35.196 fluid oz or 1.7598 pints
1 gallon	=	4.546 litres
1 meter	=	39.37 inches

°F into °C (and vice versa) :

To convert °F into °C : Subtract 32 and multiply by 5/9 ;To convert °C into °F : Multiply by 9/5 and add 32.

To convert mg/100 ml into milliequivalents and vice versa :

$$\text{Milliequivalents} = \frac{\text{mg per 100 ml x valency x 10}}{\text{molecular weight}}$$

$$\text{mg per 100 ml} = \frac{\text{milliequivalents x molecular weight}}{\text{valency x 10}}$$

Length

1 Meter	=	1.0936 yards, 3.2808 feet or 39.3701 inches
1 yard	=	0.9144 M
1 foot	=	0.3048 M
1 inch	=	25.40 mm
1 kilometer	=	0.6214 mile
1 mile	=	1.6093 kilometers

Weight

Pounds	Kilograms	Pounds	Kilograms
1	0.45	9	4.08
2	0.91	10	4.45
3	1.36	20	9.07
4	1.81	30	13.61
5	2.27	40	18.14
6	2.72	50	22.68
7	3.18	100	45.36
8	3.63		

Kilograms	Pounds	Kilograms	Pounds
1	2.20	9	19.84
2	4.41	10	22.05
3	6.61	20	44.09
4	8.82	30	66.14
5	11.02	40	88.19
6	13.23	50	110.23
7	15.43	100	220.46
8	17.64		

Volume

Gallons	Litres	Gallons	Litres
1	4.55	9	40.91
2	9.09	10	45.46
3	13.64	20	90.92
4	18.18	30	136.38
5	22.73	40	181.84
6	27.28	50	227.30
7	31.82	100	454.60
8	36.37		

Litres	Gallons	Litres	Gallons
1	0.22	9	1.98
2	0.44	10	2.20
3	0.66	20	4.40
4	0.88	30	6.60
5	1.10	40	8.80
6	1.32	50	11.00
7	1.54	100	22.00
8	1.76		

16. FOUR-PLACE LOGARITHMS

N	0	1	2	3	4	5	6	7	8	9	Proportional Parts 1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7
N	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

FOUR-PLACE LOGARITHMS — (Contd.)

N	0	1	2	3	4	5	6	7	8	9	Proportional Parts 1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4
N	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

PERIODIC CHART OF THE ELEMENTS

Filled Shells	Ia	IIa	IIIb	IVb	Vb	VIb	VIIb	VIII	Ib	IIb	IIIa	IVa	Va	VIa	VIIa	0
	±1 **1** **H** 1.0079 1															0 **2** **He** 4.00260 2
	+1 **3** **Li** 6.941 2-1	+2 **4** **Be** 9.01218 2-2									+3 **5** **B** 10.81 2-3	+2 −4 **6** **C** 12.011 2-4	−1 +1 +2 +3 +4 +5 **7** **N** 14.0067 2-5	−2 **8** **O** 15.9994 2-6	−1 **9** **F** 18.99840 2-7	0 **10** **Ne** 20.179 2-8
	+1 **11** **Na** 22.98977 2-8-1	+2 **12** **Mg** 24.305 2-8-2									+3 **13** **Al** 26.98154 2-8-3	+2 −4 **14** **Si** 28.086 2-8-4	−3 +5 **15** **P** 30.97376 2-8-5	+4 +6 −2 **16** **S** 32.06 2-8-6	−1 +5 +7 **17** **Cl** 35.453 2-8-7	0 **18** **Ar** 39.948 2-8-8
2	+1 **19** **K** 39.098 -8-8-1	+2 **20** **Ca** 40.08 -8-8-2	+3 **21** **Sc** 44.9559 -8-9-2	−2 −3 −4 **22** **Ti** 47.90 -8-10-2	+2 +3 +4 +5 **23** **V** 50.9414 -8-11-2	+2 +3 +6 **24** **Cr** 51.996 -8-13-1	+2 +3 +4 +6 +7 **25** **Mn** 54.9380 -8-13-2	+2 +3 **26** **Fe** 55.847 -8-14-2	+2 +3 **27** **Co** 58.9332 -8-15-2	+2 +3 **28** **Ni** 58.70 -8-16-2	+1 +2 **29** **Cu** 63.546 -8-18-1	+2 **30** **Zn** 65.38 -8-18-2	+3 **31** **Ga** 69.72 -8-18-3	+2 +4 **32** **Ge** 72.59 -8-18-4	−3 +5 **33** **As** 74.9216 -8-18-5	+4 +6 −2 **34** **Se** 78.96 -8-18-6	−1 +5 **35** **Br** 79.904 -8-18-7	0 **36** **Kr** 83.80 -8-18-8
2-8	+1 **37** **Rb** 85.4678 -18-8-1	+2 **38** **Sr** 87.62 -18-8-2	+3 **39** **Y** 88.9059 -18-9-2	+4 **40** **Zr** 91.22 -18-10-2	+3 +5 **41** **Nb** 92.9064 -18-12-1	+6 **42** **Mo** 95.94 -18-13-1	+4 +6 +7 **43** **Tc** (97) -18-13-2	+3 **44** **Ru** 101.07 -18-15-1	+3 **45** **Rh** 102.9055 -18-16-1	+2 +4 **46** **Pd** 106.4 -18-18-0	+1 **47** **Ag** 107.868 -18-18-1	+2 **48** **Cd** 112.40 -18-18-2	+3 **49** **In** 114.82 -18-18-3	+2 +4 **50** **Sn** 118.69 -18-18-4	−3 +5 **51** **Sb** 121.75 -18-18-5	+4 +6 −2 **52** **Te** 127.60 -18-18-6	−1 +5 +7 **53** **I** 126.9045 -18-18-7	0 **54** **Xe** 131.30 -18-18-8
2-8-18	+1 **55** **Cs** 132.9054 -18-8-1	+2 **56** **Ba** 137.34 -18-8-2	**57-71** See Lantha-nides	+4 **72** **Hf** 178.49 -32-10-2	+6 **73** **Ta** 180.9479 -32-11-2	+6 **74** **W** 183.85 -32-12-2	+4 +6 +7 **75** **Re** 186.207 -32-13-2	+3 +4 **76** **Os** 190.2 -32-14-2	+3 +4 **77** **Ir** 192.22 -32-15-2	+2 +4 **78** **Pt** 195.09 -32-16-2	+1 +3 **79** **Au** 196.9665 -32-18-1	+1 +2 **80** **Hg** 200.59 -32-18-2	+1 +3 **81** **Tl** 204.37 -32-18-3	+2 +4 **82** **Pb** 207.2 -32-18-4	+3 +5 **83** **Bi** 208.9804 -32-18-5	+2 +4 **84** **Po** (209) -32-18-6	−1 +5 +7 **85** **At** (210) -32-18-7	0 **86** **Rn** (222) -32-18-8
2-8-18-32	+1 **87** **Fr** (223) -18-8-1	+2 **88** **Ra** 226.0254 -18-8-2	**89-103** See Acti-nides	+4 **104** (261) -32-10-2	**105** (262) -32-11-2	**106** (263) -32-12-2										

Transition Elements

Noble Gases

KEY

Oxidation States → +1 +3 **79** ← Atomic Number

Au ← Atomic Symbol

Atomic Weight → 196.9665

Electron Configuration → -32-18-1

Lanthanides	+3 **57** **La** 138.9055 -18-9-2	+3 +4 **58** **Ce** 140.12 -20-8-2	+3 **59** **Pr** 140.9077 -21-8-2	+3 **60** **Nd** 144.24 -22-8-2	+3 **61** **Pm** (145) -23-8-2	+2 +3 **62** **Sm** 150.4 -24-8-2	+2 +3 **63** **Eu** 151.96 -25-8-2	+3 **64** **Gd** 157.25 -25-9-2	+3 **65** **Tb** 158.9254 -27-8-2	+3 **66** **Dy** 162.50 -28-8-2	+3 **67** **Ho** 164.9304 -29-8-2	+3 **68** **Er** 167.26 -30-8-2	+3 **69** **Tm** 168.9342 -31-8-2	+2 +3 **70** **Yb** 173.04 -32-8-2	+3 **71** **Lu** 174.97 -32-9-2
Actinides	+3 **89** **Ac** (227) -18-9-2	+4 **90** **Th** 232.0381 -18-10-2	+4 +5 **91** **Pa** 231.0359 -20-9-2	+3 +4 +5 +6 **92** **U** 238.029 -21-9-2	+3 +4 +5 +6 **93** **Np** 237.0482 -22-9-2	+3 +4 +5 +6 **94** **Pu** (244) -24-8-2	+3 +4 +5 +6 **95** **Am** (243) -25-8-2	+3 **96** **Cm** (247) -25-9-2	+3 +4 **97** **Bk** (247) -27-8-2	+3 **98** **Cf** (251) -28-8-2	+3 **99** **Es** (254) -29-8-2	+3 **100** **Fm** (257) -30-8-2	+2 +3 **101** **Md** (258) -31-8-2	+2 +3 **102** **No** (255) -32-8-2	+3 **103** **Lr** (260) -32-9-2

Note: Atomic weights are those of the most commonly available long-lived isotopes based on the 1973 IUPAC Atomic Weights of the Elements. A value given in parentheses denotes the mass number of the longest-lived isotope.

